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INVESTIGATIONS OF THE PROCESSES OF DISCOLORATION AND DECAY OF SUGAR MAPLE, ACER SACCHARUM, ASSOCIATED WITH FOMES CONNATUS

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INVESTIGATIONS OF THE PROCESSES OF DISCOLORATION AND DECAY OF SUGAR MAPLE, *ACER SACCHARUM*, ASSOCIATED WITH *FOMES CONNATUS*.

by

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ABSTRACT

Investigations of the Processes of Discoloration and Decay of Sugar Maple, Acer saccharum Associated with Fomes connatus.

by Terry A. Tattar

Discoloration and decay of sugar maple, Acer saccharum, associated with Fomes connatus cause severe damage resulting in economic loss. The purpose of this study was to better understand these processes in living trees. Separate investigations were conducted and the results of this research were presented as three manuscripts.

Discolored and decayed tissue associated with F. connatus in sugar maple showed a pattern of physical changes and microbial successions. The lignin to cellulose ratio was approximately the same in clear, discolored, and decayed tissue. No quantitative change occurred in the amount of total extractives in all tissues. The pH and ash concentration increased and total phenolic compounds decreased as tissues discolored and decayed. Microorganisms were in discolored tissue that showed qualitatively altered extractives and increases in pH. F. connatus was in a narrow zone of discolored tissue at the border of discolored and decayed tissue. Microorganisms were in decayed tissue that showed qualitative changes in extractives, increases in pH, and substantial decreases in amount of lignin and cellulose.

The ability of Phialophora melinii and F. connatus to tolerate and to utilize certain phenolic compounds was studied in vitro to help explain successional patterns of fungi in living trees. P. melinii, which is often isolated in advance of F. connatus in columns of discolored and decayed tissue in sugar maple, tolerated and utilized phenolic compounds

which were toxic to F. connatus. The capacity of P. melinii to alter phenolic compounds, and thereby permit growth of F. connatus, was indicated. The effects of pH, manganese concentration, nitrogen source, amount of glucose present, and means of sterilization on the growth of these fungi in media containing phenolic compounds were also investigated.

Isolates of F. connatus, P. melinii, Acrostaphylus sp., from discolored tissue, and Trichoderma viride and Mortierella sp. from decayed tissue of sugar maple were grown in liquid culture media containing sources of carbon and nitrogen found in the tissue of sugar maple. These compounds included the carbohydrates of wood and their component monosaccharides, translocation compounds from xylem sap, and storage carbohydrates. Growth was measured as oven-dried weight of mycelium. All fungi utilized the carbohydrates and nitrogen sources, except Mortierella sp. which did not utilize cellulose and xylose. Only P. melinii substantially utilized gallic acid. Breakdown of cell walls in the living tree may occur both in discolored and decayed tissue and be caused by all inhabiting fungi. Selective utilization of host components by some of these fungi may enable successful colonization of wounds and initiation of the processes of discoloration and decay.

INTRODUCTION

Discoloration and decay of sugar maple, Acer saccharum, associated with Fomes connatus, cause severe damage resulting in economic losses. The severity of these losses are explained by the slow growth rate of high quality hardwoods such as sugar maple, by the diminishing supply due to increased land development, and the increasing demand for this wood free of defects. The purpose of this study was to investigate the relationships between microorganisms and the chemical changes in discolored and decayed wood of sugar maple.

Discrete experiments were conducted and the results of this research were written in three manuscripts for publication in Phytopathology, an international journal of the American Phytopathological Society. The manuscripts are: The Sequence of Microorganisms and Changes in Constituents Associated with Discoloration and Decay of Sugar Maples, Acer saccharum, Infected with Fomes connatus; Effects of Some Phenolic Compounds on the Growth of Phialophora melinii and Fomes connatus; and Carbon and Nitrogen Metabolism of Principal Fungi Associated with Fomes connatus in Sugar Maple, Acer saccharum.

The Sequence of Microorganisms and Changes in Constituents Associated with Discoloration and Decay of Sugar Maples, Acer saccharum, Infected with Fomes connatus

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ABSTRACT

Discolored and decayed tissue associated with Fomes connatus in sugar maple showed a pattern of physical changes and microbial successions. The lignin to cellulose ratio remained approximately the same in clear, discolored, and decayed tissue. No quantitative change occurred in the amount of total extractives in all tissues. The pH and ash concentration increased and total phenolic compounds decreased as tissues became discolored and decayed. Microorganisms were in discolored tissue that showed qualitatively altered extractives and increases in pH. F. connatus was in a narrow zone of dis-

ives and increases in pH. F. connatus was in a narrow zone of discolored tissue at the border of discolored and decayed tissue. Microorganisms were in decayed tissue that showed qualitative changes in extractives, increases in pH, and substantial decreases in amount of lignin and cellulose.

Decay in living sugar maple (Acer saccharum Marsh) results from processes in which woody tissues first become discolored (8,13). The discoloration process involves host response to wounding and the activity of microorganisms. Discolored tissue may then be decayed by hymenomycetous fungi. Decay of sugar maple associated with Fomes connatus (Weinm.) Gill. causes severe structural damage resulting in economic losses (10). Previous descriptions and classifications as a white rot were made on the basis of color and texture of the decayed tissue (3,17). Analyses of tissues altered by white-rot fungi were confined mainly to decay tests on wood blocks (5,11). Moreover white-rot fungi used in these studies were mainly those that decay dead wood.

In order to better understand the nature of decay in living trees, we studied decay columns in sugar maple trees, by mapping natural wounds infected with F. connatus. This was followed by studying the relationships between microorganisms and chemical changes in discolored and decayed wood.

MATERIALS AND METHODS.--Material preparation.--Thirteen sugar maple trees 8-15 cm diam. 1.2 m above ground, bearing fruit bodies of F. connatus were cut. Logs were cut transversely through the sporophores and at 10 cm-intervals above and below until the columns of

decay and discoloration ended. The column sections were dissected aseptically and mapped for microorganisms. Chips of wood 3 x 10 mm were excised from the column and incubated on a medium consisting of 10 #/g malt extract, 2 #/g yeast extract, 20 #/liter agar (13). Approximately 600 chips were cultured.

Samples of clear, discolored, and decayed tissue were obtained from seven trees. Samples were ground to pass a 40 mesh but not a 60 mesh screen. Approximately 10 #/g of air-dried, clear, discolored, and decayed tissue were obtained from each column.

Determination of ash concentration.--One gram air-dried samples were ashed in a muffle furnace at 600 C for 24 hr. Amount of ash in the crucible was recorded as total ash/g. Extracted wood samples equivalent to 1 g oven-dried unextracted ash/g. Soluble ash was determined by subtracting unextracted ash from total ash.

Determination of amount of total extractive.--Weights of oven-dried tissue were determined from 1 g of each air-dried tissue. Samples were extracted successively with alcohol-benzene (1:2, v/v) and alcohol in a soxhlet apparatus, and then in a boiling water bath (1). Amount of total extractives was calculated as wt. loss from unextracted tissues that were oven-dried.

Determination of total phenolic compounds.--One-gram air-dried samples of each tissue were extracted with methanol for 8 hr in a soxhlet apparatus. Each extract was brought to 50 ml and coned to 5 ml in a rotary evaporator. Extracts were centrifuged at approximately 750 g for 10 min in an International Clinical Model Centrifuge and decanted. Total phenolic compounds were determined on a 0.5 ml sample by the

Folin-Ciocalteu total phenol method (9).

Determination of lignin and cellulose.--Lignin content was determined on extracted wood samples equivalent to 1 g oven-dried unextracted tissue by the 72% sulfuric acid method (2). Cellulose content was calculated by subtracting amounts of lignin and insoluble ash from sample weights of extractive free tissue.

Determination of pH and specific gravity.--One gram samplesoof air-dried tissues ground to pass a 20 mesh screen were placed in 5 ml deionized water for 1 hr and the pH measured on a Beckman pH meter (17). Approximately 4-cc sections of each tissue were placed in distilled water in graduated cylinders and repeatedly aspirated until refusal. Displacement volume was recorded. Tissue were oven-dried and sp gr was calculated as the ratio of oven-dried wt of tissue to displacement volume.

Organisms isolated from columns of discolored and decayed tissue.--Very few organisms were isolated from clear tissue. Bacteria and nonhymenomycetous fungi, mostly of the genera Phialophora and Acrostaphylus, were isolated from discolored tissue in advance of F. connatus. F. connatus was isolated in a narrow band of discolored tissue at the border of decayed and discolored tissue (Fig.1). Bacteria, Actinomycetes, and nonhymenomycetous fungi, Trichoderma viride Pers. ex Fries and Mortierella sp. were isolated commonly from decayed tissue behind F. connatus. Nematodes (Rhabdites sp.), and black carpenter ants (Camponotus sp.) were common in tissue in an advanced state of decay. Microorganisms from these columns associated with F. connatus were found in a successional pattern as described for sugar maple (14,17).

Characteristics and composition of clear, discolored and decayed sugar maple tissue.-- The chemical composition of clear, discolored and decayed tissues is given in Table 1. Specific gravity of clear tissue (0.6), did not change measurably in discolored tissue but decreased to 0.25 in decayed tissue. These data indicate a weight loss of 42% per unit volume in decayed tissue. Chemical constituent values were adjusted for decayed tissues to account for the noted changes in sp gr, allowing comparisons on a constant tissue volume basis. (Table 1). The pH of discolored (6.4) and decayed (6.6) tissues was significantly greater than that of clear tissues (5.5).

Ash.-- Amount of total, insoluble, and soluble ash per unit volume increased significantly from 3, 2, and 1 mg, respectively, in clear tissue to 11, 7, and 4 mg in discolored tissue and 22, 14, and 9 mg in decayed tissue. The increase in total ash concentration from clear to decayed tissue was 18x based on weight and 8x based on volume. An increase in mineral content was shown to be associated with the processes of discoloration and decay in sugar maple (15).

These increases in soluble and bound mineral constituents could affect the utilization of other wood constituents in several ways. The concn of inorganic salts can affect the swelling of cellulose miscelles in the cell walls (18). Such swelling could affect the activity of cellulases and other wall-attacking enzymes. The mineral constituents can alter pH (8), which increased significantly from 5.5 in clear to 6.4 in discolored and 6.6 in decayed tissue, and could affect the activity of enzymes. Changes in the concn of an essential cofactor might also be important (11).

Total extractives.-- Amount of total extractives per unit volume of clear tissue did not change significantly in discolored and decayed tissues. Although the amount of total extractives appeared relatively constant there were changes in the composition of extractives during discoloration and decay.

One part of the total extractives which changed radically was that portion comprised of ash constituents. Soluble ash comprised 28% of the total extractives of decayed tissue compared to 2% of clear tissue. Another part of the extractives which changed radically was the phenolic component. Total extractable phenolic compounds per unit volume decreased from 7 mg in clear to 2 mg in discolored and were negligible in decayed tissue. In clear tissue phenolic compounds appeared to comprise 15% of the total extractives, whereas they comprised only 4% in discolored tissue. Total phenolic content was determined from methanolic extracts. A similar pattern of phenolic content was shown by results of extracts from the same tissues which had been extracted with ethanol-benzene (1:2,v/v).

The observed decrease in phenolic consititucnts may be important to the invasion of F. connatus. In vitro growth studies have shown that isolates of Phialophora melinii were able to grow at concn of certain phenolic compounds which did not permit the growth of F. connatus, and the ability of P. melinii to utilize and to alter those inhibitory compounds was demonstrated (16). Because of the high frequency of isolation of Phialophora sp. in advance of F. connatus it is possible that alteration of phenolic constituents by Phialophora sp. or other nonhymenomycetous fungi allowed F. connatus to invade discolored tissues.

Lignin and cellulose.-- Amount of lignin per unit volume increased significantly from 144 mg in clear to 174 mg in discolored and decreased significantly to 77 mg in decayed tissue. Amount of cellulose per unit volume decreased significantly from 806 mg in clear to 769 in discolored and 290 mg in decayed tissue. The ratio of cellulose to lignin remained approximately 5:1 in all tissues.

The increase in lignin could be explained by the bacteria and non-hymenomycetous fungi attacking nonlignified cellulose in the discolored tissue. Phialophora and other Fungi Imperfecti cause soft-rot in wood products by attacking non-lignified cellulose in the cell walls (7). Several isolates of two species of Phialophora utilize cellobiose at rates equal to or greater than D-glucose (4). This decrease in cellulose could be too small to be measured in the sp gr method used in this experiment and be sufficient to account for an apparent increase in lignin conen. Alteration in wall structure caused by nonhymenomycetous fungi and bacteria in advance of F. connatus may make the wall more susceptible to its attack. Discolored and decayed tissue differed by 56% in lignin per unit volume and 64% in cellulose per unit volume. There was no significant change in the lignin to cellulose ratio which suggests that lignin and cellulose are utilized at a similar rate.

Another possible explanation of the noted increase in the amount of lignin is that lignin or lignin-like substances actually increased during the process of discoloration. Increases in the amount of lignin have been reported as a resistance mechanism (6). The colored substances formed during the discoloration process may act as sulfuric acid lignin.

F. connatus, which was isolated most frequently in the boundary zone between discolored and decayed tissue is probably the primary organ-

ism which attacks the lignified cellulose of the cell wall. F. connatus appears to attack effectively both lignin and cellulose. However, micro-organisms such as T. viride, found in the decayed tissues, can produce cellulases (11). While the primary role of F. connatus may be to degrade the lignin in cell walls, degradation of cellulose in advanced decay may be due to several extracellular cellulase systems.

TABLE 1. Characteristics and composition of clear, discolored, and decayed sugar maple tissue.

Constituent	Condition of Tissue			
	Clear	Discolored	Decayed	Decayed [b]
	Mg/g oven-dried weight [a]			
Total ash	3a	11b	54c	22
Soluble ash	1	4	22	9
Insoluble ash	2a	7a	32b	14
Total extractives	46a	47a	98b	41
Total phenolic compounds	7a	2b	0.3	0.1
Sulfuric acid lignin	144a	174b	184b	77
Cellulose	806a	767b	697b	290

a Different letters designate significant differences at the 5% level of significance.

b Decay values corrected for difference in specific gravity for comparisons based on weight per unit volume.

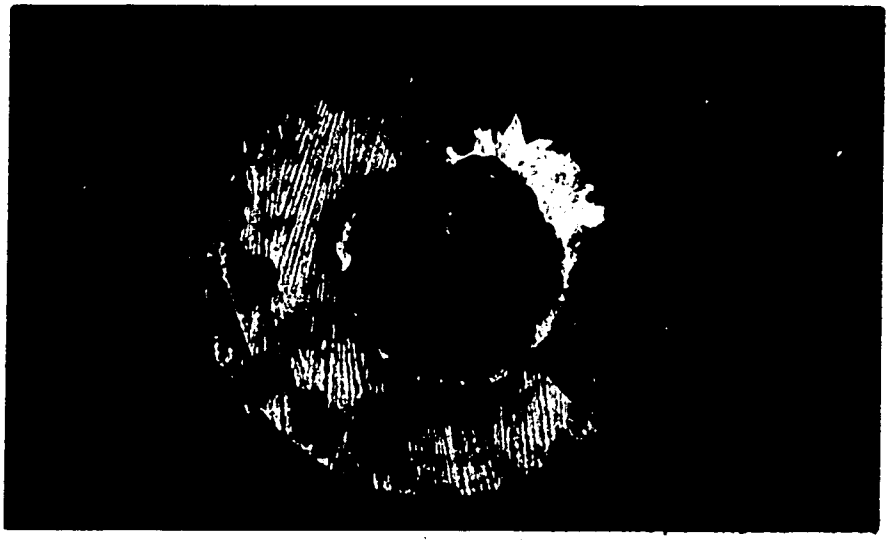

Figure 1. Decay column cross section of sugar maple showing Fomes connatus in a thin band of discolored tissue at the decay border after 1 wk incubation at 25 C.

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Effects of Some Phenolic Compounds on the Growth of Phialophora melinii and Fomes connatus.

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ABSTRACT

The ability of Phialophora melinii and Fomes connatus to tolerate and to utilize certain phenolic compounds was studied in vitro to help explain successional patterns of fungi in living trees. P. melinii, which is often isolated in advance of F. connatus in columns of discolored and decayed tissue in sugar maple, tolerated and utilized phenolic compounds which were toxic to F. connatus. The capacity of P. melinii to alter phenolic compounds, and thereby permit growth of F. connatus, was indicated. The effects of pH, manganese concentration, nitrogen source, amount of glucose present, and means of sterilization on the growth of these fungi in media containing phenolic compounds were also investigated.

Phialophora melinii (Nannf.) Conant is commonly isolated from discolored and decayed tissues of sugar maple, Acer saccharum Marsh., and other species of deciduous hardwoods (12, 13, 14). Studies of the physiology of P. melinii and Polyporus glomeratus, a decay fungus often associated with P. melinii, indicated that P. melinii grew well in culture on levels of certain phenolic compounds that inhibited the growth of P. glomeratus (W. C. Shortle. Unpublished data.). The inhibitory effect of phenolic compounds on some decay fungi has been demonstrated in culture and in living trees (8, 11). The role of phenolic compounds in host-pathogen interactions is well known (7).

The apparent differences between non-hymenomycetous fungi, such as P. melinii, and decay fungi with which they are associated, in their ability to utilize phenolic compounds could help account for successional relationships in living trees. It has been suggested that Trichocladium canadense, another non-hymenomycetous fungus which is often found in advance of decay fungi, utilizes the oxidized phenolic compounds in discolored wood in advance of Fomes igniarius (6).

Our purpose was to determine the ability of P. melinii to utilize and to detoxify phenolic compounds that inhibit or prevent the growth of Fomes connatus (Weinm.) Gill., an important decay fungus on sugar maple.

MATERIALS AND METHODS. -- Two isolates of Phialophora sp., identified as P. melinii, were used. The isolates came from

discolored tissue associated with the decay of Fomes connatus in sugar maple. The isolate of F. connatus (RLG-5660-S-SP) used came from the U. S. Forest Disease Laboratory, Laurel, Md.

The basic medium used to test the growth of P. melinii and F. connatus contained 10 g/liter D-glucose, 2 g/liter asparagine, and a basal medium of salts, buffer, trace elements, and vitamins as described by Lilly and Barnett (9). Phenolic compounds were substituted as carbon sources for all or part of the D-glucose at a rate equivalent to 10 g/liter D-glucose (4 g/liter carbon). The fraction of the carbon source comprised by a phenolic compound was reported as the ratio of that compound to glucose based on the wt of carbon.

NH_4NO_3 or KNO_3 was substituted for asparagine in some media at a rate equivalent to 2 g/liter asparagine (.4 g/liter nitrogen). Yeast extract was used in some media at a rate of 2 g/liter.

In treatments where manganese was added, a stock solution of $MnSO_4.H_2O$ was used. The stock solution was added as part of the distilled water used to make the medium so that the final concn of Mn^{++} of the treatment solution was 10, 100, or 500 ppm.

The pH of each medium was adjusted to 4.5 or 6.0 using 5N NaOH or 5N HCl. The fungi were grown in 25 ml of medium in 250-ml Erlenmeyer flasks. Sterilization was done by autoclaving at 15 p.s.i. and 121 C for 15 min or by filter sterilization using a Seitz filter. Seitz filtration gave results comparable to those using millipore filtration. The sterilized media were inoculated

with mycelium chopped 5 sec from 12 day cultures of P. melinii or 21 day cultures of P. connatus.

After incubation at 25 C for various lengths of time, depending on the rate of growth, the mycelium was harvested and oven-dried at 105 C for 24 hr in tared 10-ml beakers. Growth was reported as oven-dried wt of mycelium.

Regrowth medium. -- To determine the ability of P. melinii to alter the inhibitory properties of phenolic compounds, P. melinii was grown 14, 18, and 22 days on the basic medium in which gallic acid had been substituted for D-glucose. The mycelium was removed by filtration, and the culture medium was resterilized by filter sterilization. This resterilized solution was mixed with a glucose solution to yield a gallic acid to glucose ratio equivalent to those used to test the growth of P. melinii and P. connatus. The ratio was based on the original gallic acid content of the treatment solution which contained 4 g/liter carbon.

RESULTS. -- P. melinii utilized gallic acid as a carbon source (Table 1). It grew on all media at pH 4.5 containing gallic acid as a carbon source. At this low pH level, the gallic acid was not oxidized to the dark colored oxidation products that formed in media at pH 6.0. However, as P. melinii grew on the gallic acid medium, the color did change from light to dark which indicated the oxidation of gallic acid to products of unknown nature. This change was accompanied by an increase in pH.

There was no significant difference in growth whether the medium was autoclaved or filter sterilized which indicated that

autoclaving did not alter the response to gallic acid. The growth of P. melinii on media containing inorganic nitrogen sources in which the only carbon was in gallic acid clearly indicated the ability to utilize gallic acid. Adding high amounts of manganese increased the growth significantly.

P. melinii failed to grow on all media containing gallic acid as a carbon source at pH 6.0, unless high concn of manganese were present. It did not grow at pH 3.3. Dark colored oxidation products produced at the high level of pH were apparently toxic to P. melinii. The toxic action was nullified when high concns of manganese were present. The dark colored products of tannic acid did allow growth so they may be less toxic.

Toxic effects of the oxidation products at pH 6.0 were nullified by the presence of another carbon source, glucose, even at relatively low concns (Table 2). The growth at both levels of pH was greater as the glucose increased. At the low level of gallic acid there may be a stimulatory growth effect.

P. melinii tolerated and grew well in the presence of several phenolic compounds (Table 3); whereas similar rates were toxic to F. connatus. The growth of F. connatus was completely inhibited by all compounds except tyrosine which caused some inhibition. The mean oven-dried wt of mycelium at 25 days for F. connatus on glucose alone plus basal medium was 137 mg. When tyrosine was added at the rate of 1:10 and 1:1, growth was decreased to 55 and 66 mg, respectively.

The data indicated that ortho-dihydroxyphenolic compounds, such as catechol, are much more inhibitory to P. melinii than

meta- or para-dihydroxyphenols, such as resorcinol and hydroquinone, respectively. At the low rate, 1:10, only cinnamic acid and pyrogallol completely inhibited the growth of P. malinii. The growth on the phenolic amino acid, tyrosine, and the trihydroxy-phenolic acid, gallic acid, was not significantly different from that on glucose alone. Good growth was obtained on both resorcinol and hydroquinone. Phenol, catechol, and coumarin were inhibitory. At the high rate, 1:1, all the compounds except tyrosine, resorcinol, hydroquinone, and gallic acid were completely inhibitory to P. malinii. Of these compounds, resorcinol and hydroquinone, were strongly inhibitory.

Poor growth of F. connatus on gallic acid medium has been reported as a characteristic of the fungus (4,5,10). F. connatus did not grow when gallic acid was added alone or with glucose in a rate of 1:1 or 1:10 to the basal medium at pH 6.0 or 4.5 whether asparagine or yeast was used as a nitrogen source. Using yeast as a nitrogen source, instead of asparagine, greatly increased the growth of F. connatus when glucose was used alone; but the fungus still failed to grow in the presence of gallic acid. The mean oven-dried wt of mycelium at 25 days was 33 mg at pH 6 and 5 mg at pH 4.5 when asparagine was used and 135 mg at pH 6 and 152 mg at pH 4.5 when yeast was used.

After gallic acid present in a medium was acted upon by P. malinii, F. connatus grew (Table 4).

Growth was greatest at the 2 low levels of gallic acid; but limited growth occurred at the high rate (1:1), when P. malinii had been grown on the gallic acid medium for 14 days. The action

of P. melinii undoubtedly makes many complex changes in the medium.

DISCUSSION. -- This study has indicated the potential of Phialophora melinii not only to tolerate and utilize certain phenolic compounds which are toxic to Fomes connatus but to alter the compounds so they are no longer toxic. The total phenolic content of clear sugar maple tissue was shown to be 3X that of discolored tissue from which P. melinii was isolated (16). It is this discolored tissue in living trees which is invaded by decay fungi. As clear tissues are discolored, the pH increases (6, 14). As the dark pigments are producted in the gallic acid medium by P. melinii, there is also an increase in pH.

The ability of Phialophora spp. to utilize a wide range of nitrogen and carbon sources and to grow over a wide range of pH has been demonstrated (1). The presence of vitamins and unknown substances soluble in alcohol, acetone, ether, and chloroform extracted from wood were stimulatory to the growth of Phialophora spp. (2, 3). Phenolic compounds could have been present in these fractions. All these factors could make Phialophora spp. a primary invador with which decay fungi could not compete.

It should be noted that the apparent oxidation of gallic acid before inoculations produced a toxic or inhibitory effect on the growth of P. melinii. Early discolorations in the living tree may be caused by host response to wounding through which the phenolic compounds present are oxidized to a more toxic state. If the reaction is sufficiently strong, invasion may be prevented or inhibited. Changes in manganese concn in living trees (15)

could help overcome the toxic effects observed in culture. The carbohydrate level could also be important.

The capacity of non-hymenomycetous fungi and decay fungi to respond to different levels of various phenolic compounds probably varies greatly. Preliminary studies by the authors on other decay fungi have shown that Fomes igniarius can tolerate low levels of phenolic compounds, while Polyporus glomeratus behaves much like F. connatus. The differences in the capacity of decay fungi to tolerate certain phenolic compounds may help account for differences observed in the ability of several of these fungi to degrade wood in various stages of discoloration and decay (14).

TABLE 1. -- Growth of Phialophora melinii on gallic acid under varying conditions of pH, manganese concentration, and method of sterilization.

Treatment[a]	pH initial	pH final	Color initial	Color final	Days of growth	mean[b] oven-dried wt of mycelium (mg)
Glucose						
	6.0	4.6	--	--	8	81 ± 17
	6.0	7.9	--	--	12	116 ± 6
	4.5	6.8	--	--	8	121 ± 5
Gallic acid	6.0	-	Dark	Dark	12	0
filter sterilized[c]	6.0	-	Dark	Dark	12	0
	4.5	8.1	Light	Dark	14	61 ± 8
filter sterilized	4.5	8.6	Light	Dark	14	59 ± 3
	3.3	-	Light	Light	12	0
Gallic acid with inorganic N source						
NH_4NO_3	6.0	-	Dark	Dark	12	0
	4.5	7.6	Light	Dark	14	48 ± 3
KNO_3	4.5	8.5	Light	Dark	14	43 ± 2
Gallic acid with manganese added						
10 ppm Mn++	6.0	-	Dark	Dark	12	0
100 ppm Mn++	6.0	6.2	Dark	Dark	12	2[d]
500 ppm Mn++	6.0	7.0	Dark	Dark	12	46 ± 5
500 ppm Mn++	4.5	7.9	Light	Dark	14	86 ± 4
Tannic acid	6.0	5.4	Dark	Dark	12	22 ± 3

a Treatment refers to compounds added to a basal medium of asparagine or an inorganic N source, buffer, salts, trace elements, and vitamins (9).

b Means based on 6 observations (3 replications/isolate per treatment). The 95% confidence limits are reported for each treatment mean.

c All treatments were autoclaved at 15 psi and 121 C unless noted otherwise.

d Mean based on 3 observations because only one isolate grew at this level of manganese.

TABLE 2. -- Growth of Phialophora melinii under varying concentrations of gallic acid and glucose

Ratio of gallic[a] acid to glucose	pH initial	pH final	Color initial	Color final	Days of growth	Mean[b] oven-dried wt of mycelium (mg)
Glucose alone	6.0	4.6	--	--	8	81 ± 17
1:10	6.0	5.7	Dark	Dark	8	122 ± 10
1:1	6.0	5.6	Dark	Dark	8	92 ± 17
1:1 filter sterilized[c]	6.0	4.9	Dark	Dark	8	39 ± 4
10:1	6.0	6.9	Dark	Dark	12	33 ± 4
20:1	6.0	7.0	Dark	Dark	12	21 ± 3
Glucose alone	4.5	6.8	--	--	8	121 ± 5
1:10	4.5	6.5	Light	Dark	8	138 ± 2
1:1	4.5	6.2	Light	Dark	8	104 ± 4

a Ratio of gallic acid to glucose based on the wt of carbon added to a basal medium of asparagine, buffer, salts, trace elements, and vitamins (9).
b Means based on 6 observations (3 replications/isolate per treatment). The 95% confidence limits are reported for each treatment mean.
c All treatments were autoclaved at 15 psi and 121 C unless noted otherwise.

TABLE 3. — Growth of Phialophora melinii on various phenolic compounds

Compound[a]	Mean[b] oven-dried wt of mycelium (mg.) Ratio of compound to glucose: P. melinii[c] 1:10	1:1
Glucose alone	135	
Tyrosine	135	113
Phenol	44	0
Catechol	9	0
Resorcinol	116	20
Hydroquinone	111	8
Pyrogallol	trace	0
Gallic acid	135	102
Cinnamic acid	0	0
Coumarin	25	0

a compounds added with glucose in a ratio of 1:10 or 1:1 based on wt of carbon to a basal medium of asparagine, buffer, salts, trace elements, and vitamins (9).

b Means based on 3 replications/isolate per treatment.

c Growth at 9 days. Initial pH 4.5.

TABLE 4. — Growth of Fomes connatus on gallic acid medium after the growth of Phialophora melinii

Ratio of gallic[a] acid to glucose	Mean[b] oven-dried wt of mycelium (mg) N source: Days of growth of P. melinii 14	18	22
Glucose alone	48 ± 5	50 ± 9	53 ± 9
1:20	19 ± 17	15 ± 3	18 ± 1
1:10	26 ± 13	25 ± 7	33 ± 4
1:1	5 ± 8	0	0
Gallic acid alone	0	0	0

a Ratio based on wt of carbon of gallic acid before growth to glucose added after growth to basal medium of asparagine, buffer, salts, trace elements, and vitamins (9).

b Means based on 4 replications/isolate per treatment. The 95% confidence limits are reported for each treatment mean. Growth at 25 days.

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Carbon and Nitrogen Metabolism of Principal Fungi Associated with *Fomes connatus* in Sugar Maple, *Acer saccharum*.

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ABSTRACT

Isolates of *Phialophora melinii* and *Acrostaphylus* sp. from discolored tissue, *Trichoderma viride* and *Mortierella* sp. from decayed tissue and *Fomes connatus* from a narrow band of discolored tissue at the border of decayed and discolored tissue, of sugar maple (*Acer saccharum*) were grown in liquid culture media containing sources of carbon and nitrogen found in tissue of sugar maple. These compounds included the carbohydrates of wood and their component monosaccharides and translocation compounds from xylem sap. Growth was measured as oven-dried weight of mycelium. All fungi utilized the carbohydrate and nitrogen sources, except *Mortierella* sp. which did not utilize cellulose or xylose. Only *P. melinii* substantially utilized

gallic acid. The degradation of cell walls in living trees may occur both in discolored and decayed tissue and may be caused by nonhymenomycetous and hymenomycetous fungi. Selective utilization of host components by some of these fungi may enable successful colonization of wounds and initiation of the processes of discoloration and decay.

Discoloration and decay of woody tissues involve complex changes in their composition and these changes have been associated with nonhymenomycetous and hymenomycetous fungi (12,14). The principal fungi associated with Fomes connatus (Weinm.) Gill in columns of discolored and decayed tissue of sugar maple, Acer saccharum Marsh., were Phialophora melinii (Nannfeldt) Conant, Acrostaphylus sp., Trichoderma viride Pers. ex. Fr., and Mortierella sp. (14). In that study P. melinii and Acrostaphylus sp. were isolated consistently from discolored tissue in advance of F. connatus, which was isolated from a narrow band of discolored tissue contiguous to the border of decayed tissue.

The purpose of this experiment was to obtain additional information about the role played by the principal fungi in processes of discoloration and decay through physiological studies. We studied the effect of compounds that are found in the host tissues on the growth of the principal fungi associated with these processes.

MATERIALS AND METHODS.-- Sources of carbon and nitrogen found in sugar maple or woody tissue in general were selected for these studies. These compounds included the carbohydrates in woody tissue and translocation compounds from xylem sap of sugar maple. Several forms of cellulose and the component monosaccharides of cellulose and the hemicelluloses were provided.

Isolates of F. connatus, P. melinii, Acrostaphylus sp., T. viride, and Mortierella sp. were selected at random from isolates obtained during another study on sugar maple (14). Fomes connatus isolate SP5660-s came from the U.S. Forest Disease Laboratory, Laurel, Maryland.

The medium used to test growth of these fungi contained 10g/liter D-glucose, 2g/liter L-asparagine, and a basal medium of salts, buffer, trace elements, and vitamins (8). Compounds were substituted as a carbon source for the D-glucose at an amount equivalent to 10g/liter D-glucose (4g C/liter). Compounds were substituted as a nitrogen source for L-asparagine at an amount equivalent to 2g/liter asparagine.(.4g N/liter). Yeast extract was used in some media at a rate of 2g/liter.

The pH of each medium was adjusted to 6.0 using 5N NaOH or 5N HCl. The fungi were grown in 25ml of medium in 250ml Erlenmeyer flasks. Sterilization was by autoclaving at 15 p.s.i. and 121C for 15 minutes. The carbohydrates were autoclaved separately and added aseptically to the medium after it cooled. Separate experiments were conducted using media sterilized by Seitz and Millipore filtration.

RESULTS AND DISCUSSION.-- Sterilization by either Seitz or Millipore filtration retarded growth of the fungi with most carbohydrates. In no case did filter sterilizing yield greater growth than autoclaving.

All fungi utilized, individually, the component monosaccharides of wood as their only carbon source, except Mortierella sp. which did not utilize xylose. Hemicelluloses are composed of mixed polymers of these monosaccharides (2) and were found to comprise 14.6% of the oven-dried wt of the tissue of sugar maple (10). These compounds in living trees could be attacked by P. melinii, Acrostaphylus sp., and F. connatus in discolored tissue, and by

T. viride and Mortierella sp. in decayed tissue. Liese (7) has postulated that the degradation of encrusting substances like hemicelluloses increases the accessibility of cellulose to enzyme attack.

All fungi except Mortierella sp. utilized some form of cellulose (Table 1). Phialophora melinii and Acrostaphylus may utilize nonlignified cellulose in discolored tissue in living trees because some species of Phialophora can incite soft rot in wood products (4). Fomes connatus may utilize both lignified and non-lignified cellulose in discolored tissue. Trichoderma viride may utilize non-lignified cellulose in decayed tissue. Because all other fungi that utilized cellulose did so more rapidly than did F. connatus, the role of F. connatus in the total utilization of cellulose in trees may be limited. Wood block studies with sugar maple revealed that F. connatus did not cause substantial weight loss in clear, discolored, or decayed tissue after 1 month incubation in the dark at 25 C. (T. A. Tattar 1969. Unpublished data.) Liese (7) reported that hymenomycetous fungi associated with white rot of wood start to metabolize lignin and hemicelluloses, initially, and degrade cellulose at a later stage and postulated that this phenomenon could explain the slow rate of decomposition of tissues associated with white rot hymenomycetous fungi.

All the fungi were able to utilize sucrose, starch, and maltose. The utilization of sucrose, a major component of xylem sap of sugar maple (5,15), would be advantageous for rapid colonization of wounds and for spore germination. Starch and maltose, storage and storage breakdown products of parenchymatous tissue, would also serve as energy sources at this time.

All the fungi utilized the nitrogenous compounds which have been found previously in zylem sap (1,9) as their only nitrogen source. Because woody tissues contain very little nitrogen (3) these compounds in xylem sap may be

important in meeting the nitrogen requirements of wood-inhabiting fungi. Utilization of the major nitrogenous compounds of xylem sap may enable these fungi to compete successfully with other microorganisms in the processes of discoloration and decay.

Only P. melinii was able to utilize gallic acid effectively as a sole carbon source. Phenolic compounds were found in sugar maple tissue (14) and the inhibitory effect of these compounds on decay fungi has been demonstrated in culture and in living trees (6,11). Phialophora melinii utilized gallic acid, altered the gallic acid medium, and later permitted the growth of F. connatus (13). Phialophora melinii, because of its ability to utilize high concentrations of phenolic compounds, may be altering these compounds in the living tree and permitting the growth of the other microorganisms in succession.

From this study it can be concluded that in addition to F. connatus the associated non-hymenomycetous fungi were capable of utilizing the components of woody tissue and xylem sap of sugar maple. The actual digestion of cell walls in the living tree may occur both in discolored and decayed tissue, and be caused, in part, by all the inhabiting fungi. Only P. melinii utilized gallic acid at high concentrations, and this may suggest an essential alteration of inhibitory compounds by certain microorganisms in the discolored tissue. These fungi utilized host tissue constituents which would be present during the initial time of wounding and this could be an important factor in their presence in these tissues. The processes of discoloration and decay in living trees are not only associated with non-hymenomycetous and hymenomycetous fungi but are also associated with bacteria which undoubtedly interact with the fungi and play an important role in these processes.

TABLE 1. -- Growth of *Phialophora melinii*, *Acrostaphylus* sp., *Fomes connatus*, *Trichoderma viride* and *Mortierella* sp. on various nitrogen and carbon sources.

Compounds[1]	Mean[2] oven-dried wt of mycelium (mg)					
	P. melinii (8)[6]	*Acrostaphylus* sp. (10)	*Fomes connatus* 60-s (21)	*Fomes connatus* 62F (21)	*Trichoderma viride* (6)	*Mortierella* sp. (6)
Nitrogen[3]						
Allantoin	113 ± 12	90 ± 10	19 ± 4	28 ± 12	130 ± 12	17 ± 5
Asparagine	132 ± 4	74 ± 10	50 ± 17	23 ± 8	159 ± 5	121 ± 3
Glutamic acid	147 ± 12	76 ± 5	6 ± 4	15 ± 7	191 ± 4	158 ± 5
Yeast extract	101 ± 6	68 ± 4	137 ± 8	64 ± 26	124 ± 9	112 ± 2
Carbon[4]						
Glucose	155 ± 14	67 ± 30	137 ± 8	71 ± 25	188 ± 30	125 ± 18
Xylose	133 ± 11	66 ± 17	105 ± 8	59 ± 30	163 ± 12	-
Arabinose	88 ± 24	54 ± 12	11 ± 2	11 ± 2	95 ± 16	Trace
Mannose	103 ± 22	95 ± 4	106 ± 30	26 ± 5	182 ± 33	128 ± 40
Galactose	136 ± 40	57 ± 35	22 ± 5	16 ± 2	195 ± 12	130 ± 26
Sucrose	176 ± 22	61 ± 9	34 ± 13	28 ± 16	157 ± 18	22 ± 3
Maltose	127 ± 5	71 ± 3	26 ± 10	15 ± 7	141 ± 7	26 ± 10
Starch	83 ± 18	74 ± 10	25 ± 3	7 ± 2	142 ± 42	21 ± 2
Cellobiose	126 ± 21	70 ± 31	140 ± 29	103 ± 25	180 ± 21	Trace
Cellulose[5]	103 ± 20	Trace	92 ± 21	129 ± 15	82 ± 30	-
Methyl cellulose	Trace	Trace	26 ± 14	28 ± 20	94 ± 31	Trace
Gallic acid	63 ± 12	-	-	-	14 ± 8	-

1 Compound refers to carbon or nitrogen source added to a basal medium of buffer, salts, trace elements and vitamins (8).

2 Means based on 3 replications/isolate per treatment. The 95% confidence limits are reported for each treatment mean.

3 Glucose served as carbon source in these studies at 10g/liter.

4 All fungi except *F. connatus* were provided Asparagine at 2 g/liter as a nitrogen source. *F. connatus* was provided yeast extract 2g/liter.

5 Whatman No. 40 filter paper.

6 Days of growth. Initial pH 6.0.

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