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Role of oxalic acid in incipient brown-rot decay

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Contents

1. Introduction

A complete understanding of the processes of wood degradation by brown-rot fungi has eluded researchers for decades. This knowledge is important because enormous economic losses are incurred by the decay of wood in service.

Shortly after colonizing wood, brown-rot fungi cause a sharp and rapid reduction in degree of polymerization (DP) of holocellulose with concomitant strength loss without removing the lignin (E. B. COWLING, 1961). To

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accomplish this, fungi must produce degrading agents that penetrate rapidly into the pore structure of wood. However, research has demonstrated that hemicellulases and cellulases characterized to date cannot initiate decay because they are too large to penetrate the pores of wood (D. S. FLOURNOY et al., 1991; F. GREEN et al., 1989 b; E. SREBOTNIK and K. MESSNER, 1991). One current assumption is that brown-rot fungi produce a nonenzymatic, low-molecular-weight decay agent or agents (T. L. HIGHLEY et al., 1989). Thus, research has centered on isolating and characterizing a low-molecular-weight, nonenzymatic initiator or initiators of brown-rot decay.

Research on the mechanism of brown-rot decay has focused on the Fenton reaction (Fe: H_2O_2) (G. HALLIWELL, 1965; J. W. KOENIGS, 1974; T. K. KIRK et al., 1991), one-electron (e^-) oxidation (A. ENOKI et al., 1990, 1991) and oxalic acid production (J. BECH-ANDERSON, 1987; C. J. SCHMIDT et al., 1981; M. SHIMADA et al.. 1991; E. ESFEJO and E. AGOSIN, 1991). However, experiments to date have not been able to confirm convincingly the entire brown-rot decay process in vivo.

Many investigators have noted the similarities between acid hydrolysis and brown-rot decay (J. H. BIRKINSHAW et al., 1940; E. B. COWLING, 1961; L. F. HAWLEY and W. G. CAMPBELL, 1927; G. KEILICH et al., 1970). E. B. COWLING (1961) reported substantial declines in pH of hot and cold water extracts of sweetgum sapwood decayed by *Poria monticola* Murr. (= *Postia placenta* (Fr.) M. Lam. et Lomb.). M. W. JENNISON (1952) and E. B. COWLING (1961) postulated that hydronium ions [H_3O^+] may be able to penetrate or act upon parts of the amorphous structure of the wood that are inaccessible to the much larger cellulolytic enzymes. H. E. GRETHLEIN (1985) and G. UCAR (1990) provided evidence for acid-induced increases in porosity and subsequent penetration by cellulolytic enzymes.

The rapid depolymerization of cellulose during incipient brown-rot decay was shown to be similar to acid hydrolysis of cellulose in vitro (T. L. HIGHLEY et al., 1988; T. L. HIGHLEY and L. Murmanis, 1985; and T. K. KIRK et al., 1991). However. acid-hydrolyzed (HCl) cellulose did not exhibit the oxidized characteristics of brown-rotted cellulose (T. L. HIGHLEY, 1977; T. L. HIGHLEY et. al., 1989; T. K. KIRK et al., 1991). Also, alkali solubility of acid-hydrolyzed wood differed from that of brown-rotted wood. Alkali solubility of brown-rotted wood increased rapidly at first and then proceeded more slowly, whereas alkali solubility of acid-hydrolyzed wood was essentially constant (J. W. KOENIGS, 1974).

A. RABANUS (1939) reported that *Coniophora cerebella* Pers. in liquid culture could produce pH levels as low as 2.2 and that *Laetiporus sulphureus* (Bull. :Fr.) Murr. produced pH levels as low as 1.55 by production of oxalic acid. H. SHIMAZANO (1955) characterized brown-rot fungi by the abundant accumulation of “free” oxalic acid in culture media with resultant pH < 5.5,

whereas white-rot fungi were "non-accumulators" of oxalic acid with accompanying higher pH. H. SHIMAZANO (1955) also described an oxalate decarboxylase that is involved with the breakdown of oxalic acid and reported that this enzyme was associated only with white-rot fungi. S. TAKAO (1965) tested 47 species of basidiomycetes for the production of organic acids and concluded that there were essentially two groups. One group produced oxalic acid in the presence or absence of calcium carbonate and consisted mostly of brown-rot fungi. The other group could produce oxalic acid only in the presence of calcium carbonate and consisted mostly of white-rot fungi. The low pH maintained by brown-rot fungi may also play an important role in solubilizing and reducing iron in wood. Isolates of *Gloeophyllum trabeum* (Pers.:Fr.) Murr., which produced higher weight loss, lowered the pH, whereas isolates that produced lower weight loss actually raised the pH (J. W. KOENIGS, 1974).

Some authors have postulated a direct role for oxalic acid in the brown-rot process. C. J. SCHMIDT et al. (1981) implicated oxalic acid in nonenzymatic wood decay by brown-rot fungi; oxalic acid presumably plays a catalytic role by the direct reduction of iron, supporting the hypothesis that the Fenton reagent ($Fe^{++} + H_2O_2$) depolymerizes cellulose. J. BECH-ANDERSON (1987) postulated that oxalic acid is the agent by which brown-rot fungi hydrolyze hemicelluloses and increase the accessibility of cellulose to wood decay enzymes. M. SHIMADA et al. (1991) reported that oxalic acid concentrations of 1% (pH 1.3) and 5% decreased the viscosity of cellulose (kraft pulp). These authors hypothesized that under physiological conditions, wood cellulose may be depolymerized by oxalic acid during brown-rot decay. E. ESPEJO and E. AGOSIN (1991) reported that *G. trabeum* and other brown-rot fungi oxidized ^{14}C-labeled oxalic acid to $^{14}CO_2$ during cellulose depolymerization.

In summary, the literature shows that incipient brown-rot decay is (1) initiated by a low-molecular-weight nonenzymatic agent or agents, (2) a function of rapid hemicellulose degradation and cellulose depolymerization, (3) similar to acid hydrolysis, and (4) characterized by acid production and low pH. The objective of our study was to determine the role of acid produced during early brown-rot decay by two isolates of *Postia placenta.* The principal results of this study were (1) sufficient oxalic acid is produced by *P. placenta* to lower the pH in wood to initiate the brown-rot decay process, (2) early depolymerization of carbohydrates by brown-rot fungi in situ may be mimicked by oxalic acid in vitro, and (3) the mechanism of strength loss during fungal-induced incipient decay may be accounted for by reductions in pH associated with hemicellulose and cellulose depolymerization.

Our results support the view that acid production is the key to the initial stages of brown rot and that the hydronium ion is the diffusible, low-molecular-weight "decay agent".

2. Materials and methods

Methods were designed to measure the capacity of *P. placenta* to produce sufficient oxalic acid to lower the pH of a variety of substrates, including intact wood, to a level that results in measurable hydrolytic effects.

2.1 pH measurement

2.1.1 Monitoring of acidity

Southern pine *(Pinus* spp.) wood blocks (8 mm by 8 mm by 4 mm, 18 mm by 18 mm by 9 mm, or 18 mm by 18 mm by 18 mm) were exposed to *P. placenta* isolates (MAD 698 or ME 20) according to the ASTM soil-block procedure for estimating decay through weight loss (ASTM, 1991a) and allowed to incubate at 24°C for 28 days. The only modification of the ASTM procedure was the vertical insertion of a pH microprobe (Microelectrodes, mod. MI-410 Londonderry, NH)[2] into the wood block. The tip of the microprobe was approximately 5 mm from the bottom of the block. A strip chart recorder (Farrand Optical Company, Valhalla, NY) was used to record the output from the pH microprobe through a Corning pH meter (mod. 130).

Acid dye-indicators were prepared by incorporating 0.04% cresol-red (Aldrich # 11448) or 0.04% thymol-blue (Aldrich # 11454) in 1.5% malt/2% agar (MEA) in 90-mm petri plates according to the procedures outlined in the Society of American Bacteriologist Manual (SOCIETY OF AMERICAN BACTERIOLOGISTS, 1957). These indicators change color at pH $\leq$ 1.8 for cresol-red and pH $\leq$ 2.8 for thymol-blue. The plates were inoculated with either *P. placenta* isolate MAD 698 or ME 20 and incubated at 24°C for 14 days. Plates were monitored visually on a daily basis for dye-indicator color change.

2.1.2 Oxalic acid measurement

Oxalic acid was measured with a diagnostic kit for the determination of oxalate (Sigma Chemical Co., St. Louis, MO).

2.1.3 Growth on oxalic acid agar media

Postia placenta isolates (MAD 698 and ME 20) were inoculated at the center of 90-mm petri plates containing MEA and sufficient oxalic acid to create pH levels of 1.5, 2.0, 2.5, 3.0, 3.5, 4.0, 4.5, 5.0, and 6.0. Plates were maintained at 24°C, and radial growth was measured at 3, 6, 9, 12, and 15 days. Growth was also monitored on MEA plates not supplemented with oxalic acid.

[2] The use of trade or firm names in this publication is for reader information and does not imply endorsement by the U.S. Department of Agriculture of any product or service.

2.2 Cellulose depolymerization and determination of reducing sugars

The average DP of cellulose (purified cotton type A-600, Holden Vale Manufacturing Ltd., Haslingden, England) was measured viscometrically (E. B. COWLING, 1960) with cupriethylenediamine hydroxide solvent (GFS Chemicals, Columbus, OH) (ASTM. 1953). Reducing sugars were determined by a microadaptation of the Nelson-Somogyi assay (F. GREEN et al., 1989a).

2.3 Decay tests

Weight loss was determined according to ASTM D-2017 (ASTM, 1991a). Bending strength was evaluated with two methods. Method I was developed at the Forest Products Laboratory for this study. To obtain bending strength (modulus of rupture (MOR)). 1000 g soil (ovendry weight) was placed in 229- by 330- by 51-mm metal pans such that the center of the soil formed a 30-mm-wide median ridge that was 10 mm higher than either end. Soil moisture content was adjusted to 40%. Ten southern pine test specimens (preconditioned at 23°C/80% relative humidity (RH)) per fungus-exposure combination were then placed so that their centers rested on the soil ridge. The ends of the test specimens did not contact the soil. The metal pans were covered with aluminum foil and autoclaved at 103 kPa/121°C for 45 min. Upon cooling, each test specimen was inoculated at the center with 1 ml of mycelial suspension developed in liquid culture (1% cellobiose in mineral salts; T. L. HIGHLEY, 1973) and incubated at 23°C/70% RH for the desired period. The effects of the test on bending strength were evaluated as a simply supported beam with center-point loading and a 18 : 1 span-to-depth ratio. This test method induced maximum stress in the center of the specimen.

Method II was devised by J. E. WINANDY and J. J. MORRELL. (1991). Twelve small, clear specimens per fungus-exposure combination were exposed to fungal activity while loosely packed in vermiculite. The middle one-third of 203-mm Douglas-fir *(Pseudotsuga menziesii* (Mirb.) France) heartwood specimens was inoculated with *P. placenta* and then incubated for up to 177 days in distilled-water-saturated vermiculite. The effects of the fungi on bending strength were evaluated as a simply supported beam with a 17.5:1 span-to-depth ratio. Each specimen was tested using third-point loading, which induced a constant-moment and thereby a constant stress field throughout the entire decayed area.

2.4 High pressure liquid chromatography

2.4.1 Acid-treated wood

Five southern pine sapwood miniblocks (6.4 mm by 6.4 mm by 3.1 mm, small dimension in fiber direction) were washed with distilled water under vacuum for 24 h, then placed in 50 ml of oxalic acid or 50 ml of hydrochloric acid at varying pH under vacuum. Blocks were rocked gently for 2 weeks. The acid solutions of block extracts were air dried and reconstituted to 1 ml each with distilled water for sugar analyses by high pressure liquid chromatography (HPLC) (R. C. PETTERSEN and V. H. SCHWANDT. 1991).

2.4.2 Decayed wood bending specimens

To evaluate the fungal effects on carbohydrates of the specimens prepared by Method I, a wood wafer approximately 25-mm-long by full cross-section (9.5 by 25.4 mm) was cut from the decay zone near the mechanical failure of the specimen. The wafer was ground to 30 mesh (595 µm), and material from all tested specimens from each fungus-exposure combination was combined. This ground sample was analyzed for carbohydrates using HPLC (R. C. PETTERSEN and V. H. SCHWANDT, 1991).

2.4.3 Degraded fire-retardant-treated wood

To evaluate the effects of fire retardant treatment (FRT) and elevated temperature on MOR and carbohydrate components, FRT specimens exposed at 82°C for five specified periods up to 160 days were tested according to ASTM D-143 (ASTM. 1991 b). After flexure testing, a wafer was cut from the decay zone near the mechanical failure of the specimen. The wafer was ground to 30 mesh (595 µm), and material from all tested specimens from each fungus-exposure combination was combined. A portion of this ground sample was analyzed for carbohydrates using HPLC (R. C. PETTERSEN and V. H. SCHWANDT, 1991).

2.5 Scanning electron micrcscopy

Individual specimens were cryofixed by rapid quenching in precooled (1.4 kPa. -210°C) liquid nitrogen, followed by lyophilization without chemical fixation. The samples were dehydrated by either freeze- or air-drying. For freeze drying. cryofixed decayed southern pine blocks or hyphae on glass coverslips were transferred to a pre-cooled cryovessel and lyophilized overnight at -55°C for 12 h. Other samples were not cryofixed but air dried in a desiccator with calcium sulfate for 12 h. Specimens (wood and glass coverslips) were coated with gold in a Polaron sputter-coater for approximately 22 s, resulting in a 6.5- to 7.5-mm-thick gold layer. Specimens were examined with a Hitachi S-530 scanning electron microscope at an accelerated voltage of 25 kV and working distances between 5 and 10 mm.

3. Results

3.1 pH reduction and oxalic acid production

Figure 1 shows the change in pH of wood blocks during the first 4 weeks of exposure to *P. placenta* (MAD 698 and ME 20) in the soil-block test. Isolate MAD 698 rapidly lowered the pH to 2.5 (run 1) and 1.6 (runs 2 and 3) within 1 week (runs 2 and 3 had smaller blocks). Isolate ME 20 affected a rise in pH during the same period and stabilized above 1.0. After initial readings were measured with water supplementation, the noninoculated control block dried out, and the pH rose to 7.0. Oxalate concentration in miniblocks was shown to increase (MAD 698) over the first 5 days after inoculation (Fig. 1). Oxalate production by ME 20 was not detected over the same period (days 1 to 7).

Microprobe pH of brown-rot decay

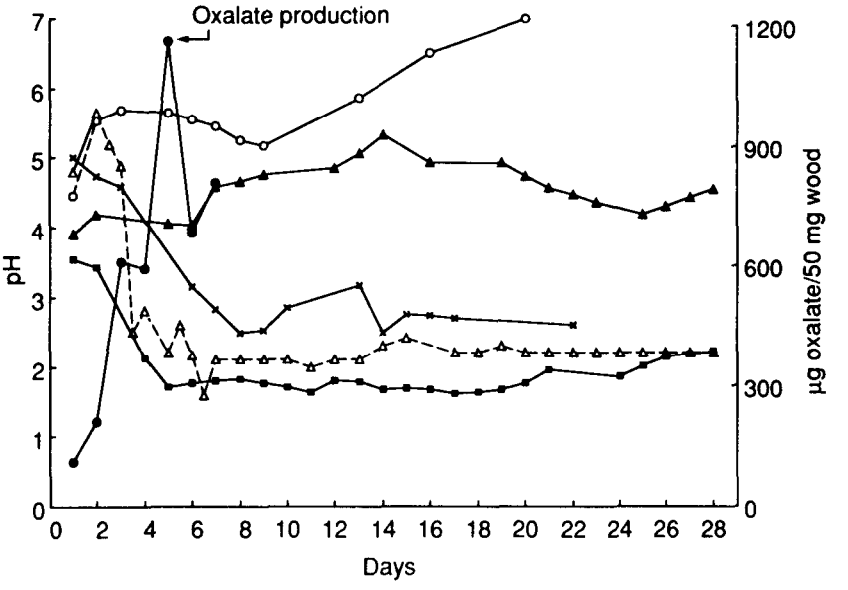

Fig. 1. Continuous pH measurement of wood blocks by microprobe of brown-rot decay by *Postia placenta* isolates (ME 20/MAD 698) in southern pine. Oxalate production by MAD 698. Legend: ○ Control, ▲ ME 20, X MAD 698 (run 1), Δ MAD 698 (run 2), ■ MD 698 (run 3).

The MAD 698 and ME 20 isolates lowered the pH of MEA supplemented with acid dye-indicators. Cresol-red (pH range 0.2 - 1.8) and thymol-blue (pH range 1.2 - 2.8) showed positive color changes with both isolates in 7 days. In addition, MAD 698 and ME 20 showed no growth inhibition on MEA supplemented with oxalic acid to pH 2.0 compared to unsupplemented 2% MEA.

The relative capacity of isolates MAD 698 and ME 20 to produce oxalic acid on exposure to a variety of sugars and woody substrates is shown in Table 1. In general, oxalic acid production correlated directly with decreases in pH of the substrates after 2 to 4 weeks of incubation on glucose or malt agar. Substrate composition influenced the production of oxalate by the two test isolates; isolate ME 20 produced similar concentrations as did MAD 698, except for xylan and chitosan substrates. Oxalate production was reduced when substrates were seeded onto glucose agar.

Table 1: Relative oxalate production by *Postia placenta* isolates on various carbohydrate substrates on coverslips over malt or glucose agar[a)]

Substrate	Initial pH	MAD 698				ME 20			
		Malt		Glucose		Malt		Glucose	
		Oxalate	pH	Oxalate	pH	Oxalate	pH	Oxalate	pH
Southern pine	4.4	64	3.5	69	3.9	368	3.0	60	3.5
Cellulose	4.8	28	4.0	17	5.0	129	3.7	5	4.3
Holocellulose	4.5	494	4.1	153	7.8	443	3.5	63	4.3
Cellobiose	–	25	3.7	–	–	228	2.7	–	–
Mannan	3.0	31	3.0	87	2.8	514	3.7	8	5.0
Xylan	6.5	1,023	4.6	656	4.0	11	7.2	8	7.2
Chitosan	7.1	1,502	1.8	4	7.3	152	5.7	3	6.0

a) Initial and final pH of 50 mg substrate in 2 cm^3 distilled H_2O. Oxalate is expressed in micrograms per cubic centimeter.

3.2 Cellulose depolymerization

The ability of aqueous oxalic acid to effect depolymerization of cotton cellulose (ground, 0.5 mm screen) in 7 days of incubation is shown in Figure 2. Depolymerization increased proportionately with a decrease in pH, especially below pH 3.0, where DP values below 200 were achieved with "saturated" oxalic acid. In spite of rapid decreases in DP, reducing sugars or glucose were not detected by micro-Nelson-Somogyi or glucose oxidase assays.

The relative capacity of MAD 698 and ME 20 to effect depolymerization of cotton cellulose by direct colonization is shown in Table 2. When cotton cellulose was placed on precolonized southern pine feeder strips in soil-woodblock tests, only MAD 698 caused measurable decreases in DP after 17 weeks. The final pH of this cellulose was 5.0 after degradation.

3.3 Acid treatment

To assess the effects of acid on wood, southern pine miniblocks were immersed in either hydrochloric acid or oxalic acid at pH range 1.0 - 4.0 for 2 weeks; acid washes were analyzed by HPLC for sugar solubilization (Table 3). At physiologically achievable pH levels for brown-rot decay by MAD 698 (Fig. 1), hemicelluloses were apparently depolymerized. Oxalic acid (pK = 1.46) did not cause as much solubilization as the mineral acid. However, decreases in extractible sugars were observed at higher pH levels and increases at lower levels.

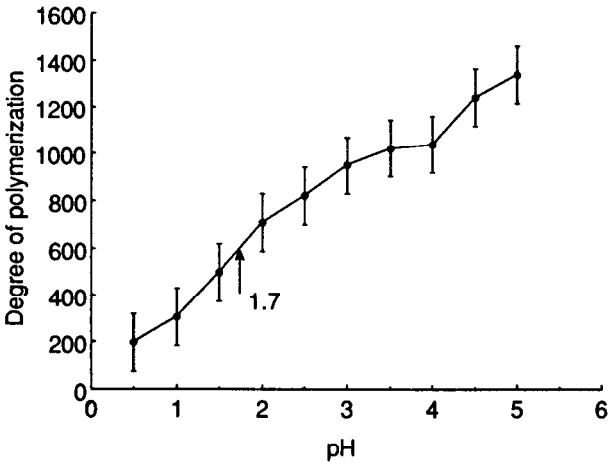

Fig. 2. Relative decrease in degree of polymerization (DP) of unrinsed cotton cellulose treated with oxalic acid and water (five samples of 50 mg/group) in 7 days at 40°C. Arrow indicates lowest pH in wood-block measurement (run 3) in Figure 1.

Table 2: Depolymerization of cellulose by *Postia placenta* isolates in soil-block test after 17 weeks

Isolate	Cellulose	
	pH	DP
ME 20	5.63	1,966
	5.66	1,766
ME 20 (with glucomannan)	5.47	1,538
	4.45	1,604
MAD 698	4.96	212
	4.91	286
Control (uninoculated)	–	1,941

3.4 Strength loss

To compare the relative strength loss of brown-rot decay by isolates MAD 698 and ME 20, southern pine wood strips were centrally inoculated with liquid cultures of each fungus and incubated under ASTM soil-wood-block conditions for 2, 4, or 6 weeks. The results of Method I showed that identical strength loss was measurable in both isolates after only 2 weeks of incubation (Fig. 3). However, weight loss was measurable only in MAD 698.

Table 3: Analysis of sugars solubilized by exposure of southern pine miniblocks for 2 weeks to oxalic acid or hydrochloric acid at various pH levels[a)]

Treatment	pH	Arabinose	Galactose	Glucose	Xylose	Mannose
Hydrochloric acid	1.0	2.81	0.39	0.23	0.21	0.11
	1.5	2.31	0.29	0.24	0.07	0.07
	2.0	0.73	0.17	0.06	0.03	0.07
	2.5	0.28	0.18	0.08	0.03	0.07
	3.0	0.16	0.07	0.04	0.02	0.06
	3.5	0.07	0.02	0.02	0.01	0.03
	4.0	0.03	0.01	0.01	0.01	0.02
Oxalic acid	1.0	0.44	0.06	0.03	0.01	0.02
	1.5	0.22	0.17	0.16	0.08	0.08
	2.0	0.18	0.14	0.13	0.06	0.06
	2.5	0.20	0.16	0.12	0.06	0.06
	3.0	0.13	0.13	0.06	0.03	0.05
	3.5	0.13	0.09	0.04	0.02	0.05
	4.0	0.10	0.08	0.04	0.02	0.06
Control (tap water)	5.0	0.08	0.04	0.03	0.02	0.04
	5.0	0.03	0.02	0.02	0.01	0.01

a) Sugars are expressed as milligrams per milliliter per group of five miniblocks.

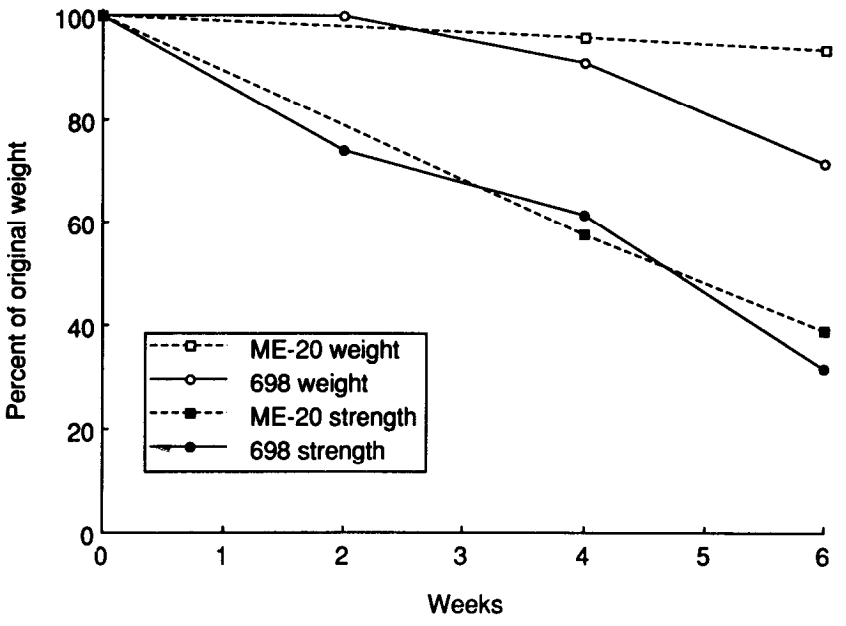

Fig. 3. Strength loss compared to weight loss of wood strips inoculated with *Postia placenta* MAD 698 and ME 20.

Figure 4 shows the relationship between strength and chemical composition of wood. The figure shows the effects of biologically induced brown-rot decay on specimens prepared by Method II (Fig. 4A) and of thermally induced acid hydrolysis at elevated temperatures on specimens treated with fire retardants (Fig. 4B). The decrease in MOR paralleled the decrease ***in*** hemicellulosic sugars, especially arabinose and galactose.

Table 4: Comparison of MAD 698 and ME 20 isolates of *Postia placenta*

Isolate characteristic	ME 20	MAD 698
Production of glucan in liquid culture[a]	No	Yes
Ability to depolymerize cellulose[b]	DP 1,600	DP 250
Minimum pH of wood blocks in soil-block test	3.9 at 5 days	1.6 at 3 days
Weight loss of southern pine in 10 weeks	5 %	50 %
Ability to produce oxalate *in vitro*	Yes	Yes
Ability to produce oxalate in wood	No	Yes
Acid dye-indicator (cresol-red)	Positive pH[c]	Positive pH[c]
Ability to cause strength loss (MOR)	Yes	Yes
Appearance of hyphal sheath	Smooth	Fibrillar
Appearance of decayed S_2 layer	Bundled	Amorphous
Production of degradative enzymes[a], [d]	Yes	Yes
Growth on oxalic acid agar (pH = 2.0)	Yes	Yes

a) J. A. MICALES et al., 1990. – b) Depolymerization at 17 weeks. – c) pH ≤ 1.8. – d) J. A. MICALES and T. L. HIGHLEY, 1989.

3.5 Ultrastructure

Scanning electron micrographs (SEMs) comparing brown-rot decay and sheath morphology of *P. placenta* (MAD 698 and MA 20) are shown in Figure 5. Figure 5a illustrates the remaining intact cellulose bundles of ME 20 and Figure 5 b the amorphous S_2 region of the wood cell wall attacked by MAD 698. The hyphal sheath matrix of ME 20 is smooth and nonfibrillar (Fig. 5c) compared with the fibrillar sheath of MAD 698 grown on xylan (Fig. 5d).

In Figure 6, SEMs compare the residual wood structure of untreated and unexposed wood, FRT (acid-treated) wood, and wood exposed to brown-rot decay (MAD 698). Figure 6b shows limited bundling of FRT wood in the S_2 layer and is comparable to Figure 5a. Also, the amorphous nature of the S_2 cell wall for both the wood decayed by MAD 698 (Fig. 6c) and FRT wood (Fig. 6d) is similar. The similarity of decayed wood and FRT wood exposed at high temperatures is apparent.

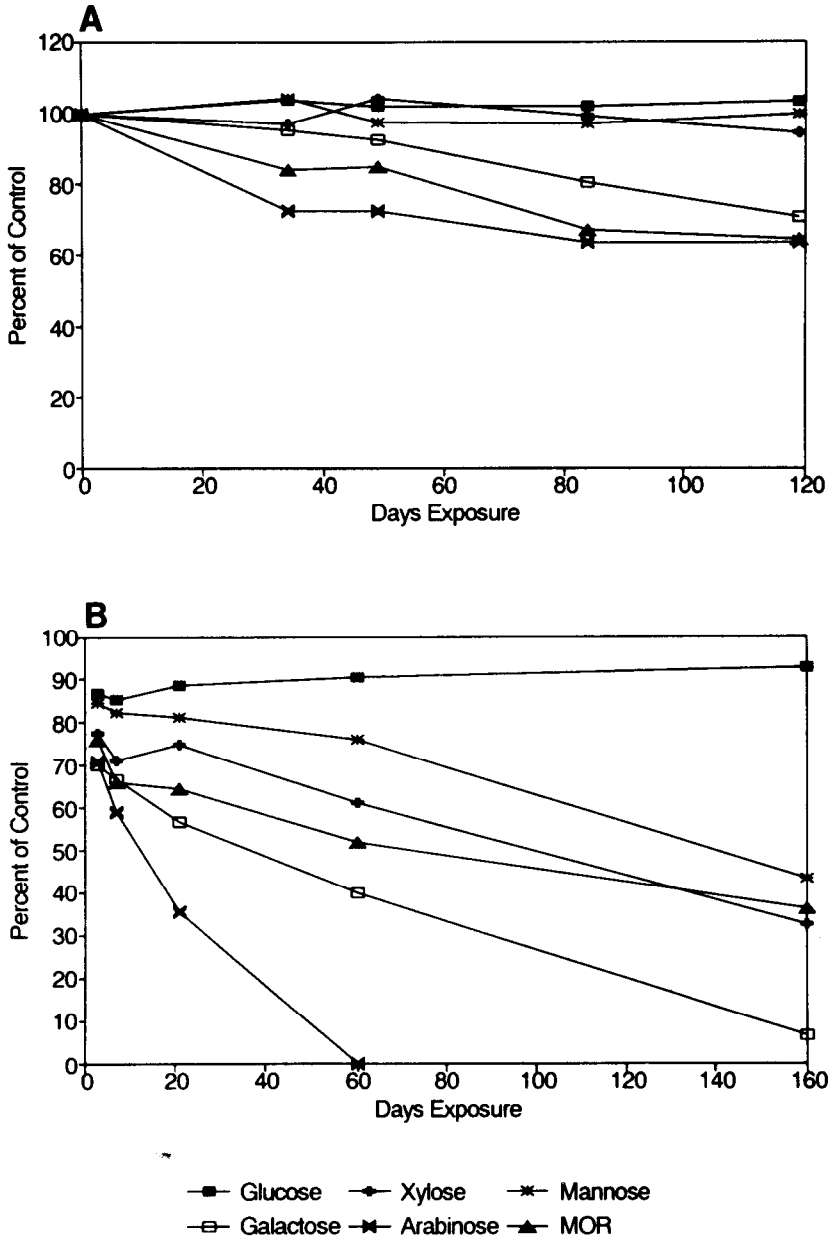

Fig. 4. Relationship between chemical composition and bending strength. A, Douglas-fir heartwood exposed to *Postia placenta* for various periods up to 178 days (adapted from J. E. WINANDY and J. J. MORRELL, submitted). B, southern pine sapwood treated with monoammonium phosphate and exposed at 82°C/50% relative humidity for various periods up to 160 days (adapted from S. L. LEVAN et al., 1990).

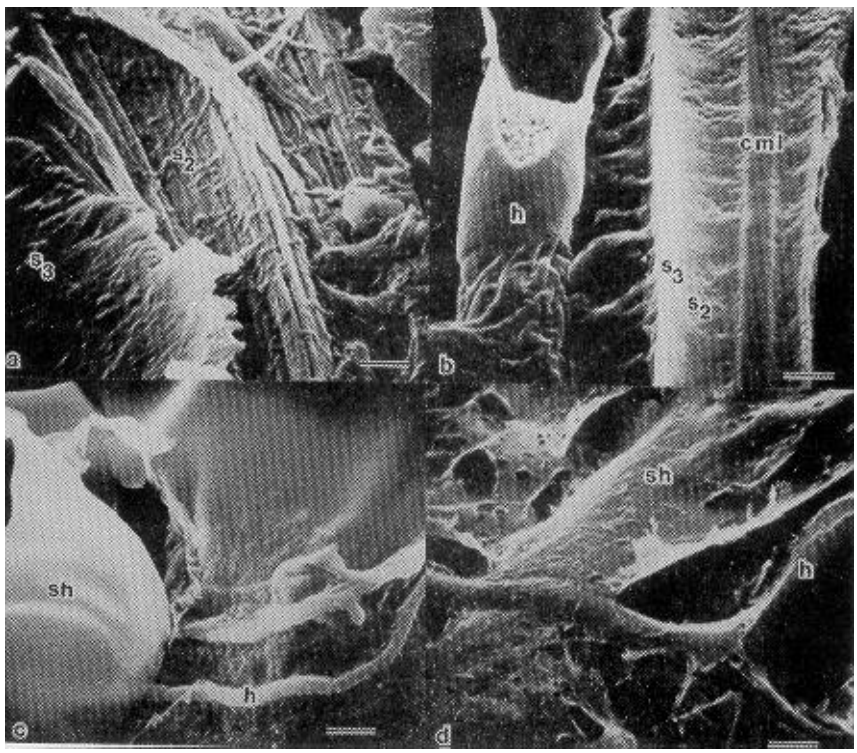

Fig. 5. Scanning electron micrographs of decayed southern pine and hyphal sheaths (sh) for *Postia placenta* isolates ME 20 (a, c) and MAD 698 (b, d). (a) bundles of cellulose fibers in S_2 layer and apparent disruption of hemicellulosic materials in wood decayed by ME 20-S_3 layer visible on left; (b) amorphous (nonbundled) wood decayed by MAD 698-hyphae (h), S_2 and S_3 layers, and compound middle lamella (cml) visible; (c) smooth hyphal sheath of ME 20; (d) fibrillar hyphal sheath of MAD 698. × 10 000. Scale bar = 1 μm.

4. Discussion

The focus of our investigations was to determine if acid production by *P. placenta* (MAD 698) is the principal means by which the fungus initiates the brown-rot decay process. Our results with *P. placenta* MAD 698 demonstrate that incipient brown-rot decay may be characterized by early acid production, which accounts for the rapid depolymerization of hemicellulose and cellulose, followed by strength loss. Results with *P. placenta* ME 20 differ from those with MAD 698. ME 20 did not cause a sharp, sustained drop in wood pH, did not produce detectable oxalate on wood, and did not depolymerize cotton cellulose. This suggests that early utilization of hemicellulose alone by ME 20, independent of oxalic acid production,

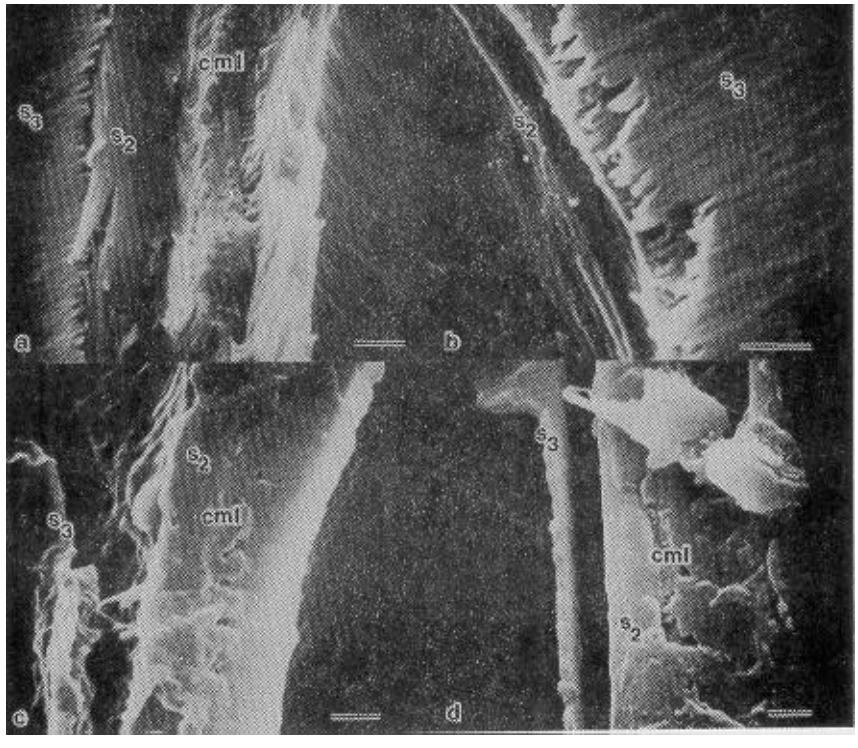

Fig. 6. Scanning electron micrographs of untreated wood (a), FRT wood (b, d), and wood decayed by *Postia placenta* isolate MAD 698 (c). The S_3 and S_2 layers and compound middle lamella (cml) are easily discernible in untreated wood (a). The FRT wood shows cellulose fibers in S_2 layer (b). Separation of S_3 layer, amorphous S_2 layer, and barely visible cml show advanced decay in wood exposed to MAD 698 (c) and advanced effects of acid in FRT wood (d). (a) and (c), × 10 000; (b), × 30 000; (d), × 8000. Scale bar = 1 μm.

accounts for the rapid strength loss that accompanies decay by this isolate (Fig. 3). An alternative hypothesis to account for early strength loss by ME 20 would be low production and rapid depletion of oxalic acid on wood by this isolate. *Postia placenta* isolate ME 20 was not defective in its ability to produce oxalic acid in vitro (Table 1); however. the substrate specificity of oxalic acid production by isolate ME 20 differed from that by isolate MAD 698 (Table 1). Isolate ME 20 was stimulated by mannan and cellobiose to produce oxalic acid, whereas isolate MAD 698 produced more oxalic acid when grown on xylan. This differential substrate effect may account in part for decay differences between these isolates. Nevertheless, isolate ME 20 could not effect weight loss of either wood or cotton cellulose, suggesting a defect in cellulose depolymerization and utilization, possibly related to a defective hyphal sheath (i.e., a nonfibrillar sheath).

Our data support the hypothesis that oxalic acid, one of the principal organic acids produced by brown-rot fungi (S. TAKAO, 1965), cleaves hemicellulose side-chains and eventually depolymerizes the hemicellulose and cellulose, causing significant strength loss prior to significant weight loss. Thus, we support the hypothesis of J. BECH-ANDERSON (1987); E. B. COWLING (1961); M. W. JENNISON (1952); N. I. NIKITIN (1966); and M. SHIMADA et al. (1991) that the elusive, low-molecular-weight, nonenzymatic, diffusible agent of incipient brown-rot decay is the hydronium ion (H_3O^+). For brown-rot decay, the hydronium ion appears to originate primarily from oxalic acid. Additional organic acids (formic, butyric, acetic, citric, etc.) either produced by the fungus or potentiated by depolymerization of hemicellulose and cellulose together contribute to the acid effect (S. ANANTHANARAYANAN and S. A. WAJID, 1970; J. H. BIRKINSHAW et al., 1940; M. W. JENNISON, 1952; and S. TAKAO, 1965).

The concentration of some oxalic acid solutions employed by J. BECH-ANDERSON (1987) (10% oxalic acid; pH ca. 0.6 by our measurement) and M. SHIMADA et al. (1991) (5% oxalic acid; pH ca. 0.8 by our measurement) appear to be too high to simulate the biological pH levels required for decay. Nevertheless, these authors did conclude that oxalic acid production was a key step in potentiating the hydrolysis of hemicelluloses, thus solubilizing low-molecular-weight sugars and increasing the accessibility of cellulose to hydrolysis.

M. SHIMADA et al. (1991) also presented pH data indicating that *Fomitopsis palustris* (Berk. et Curt.) Gilbn. et Ryv. can produce an acid environment in liquid culture of ca. pH 1.8, principally as a result of the production of oxalic acid. Our growth data on MAD 698 and ME 20 grown on oxalic acid agar at pH 2.0 demonstrate that the fungus can grow at pH $\leq$ 2.0. In concert with this is the ability of both isolates of *P. placenta* to initiate a color change from yellow to orange-red to red of the acid dye-indicator cresol-red, indicating a pH < 1.8. This low pH was verified by our experiments using a pH microprobe in brown-rot-decayed wood blocks. where the lowest pH measured was ca. 1.7. Thus, *P. placenta* (MAD 698) apparently can survive and flourish at a pH much lower than previously recorded (E. B. COWLING, 1961). Note that of all the short-chain organic dicarboxylic acids, oxalic acid is the strongest, with a pK of 1.46.

The rapid decline in pH, which is most noticeable in early decay, is interpreted as the key to subsequent events in the decay process. Although we have recorded pH levels as low as 1.7, our data show that hemicellulose components may be solubilized at higher pH (Table 3).

J. E. WINANDY and R. M. ROWELL (1984) theorized that hemicellulose is the first line of defense of wood against chemical, thermal, or biological degradation. Other authors subsequently showed that brown-rot decay and ther-

mally induced acid hydrolysis by fire retardants attack the same components (galactose, arabinose, and to a lesser extent mannose) at the onset of strength loss (Fig. 4) (S. L. LEVAN et al., 1990; J. E. WINANDY and J. J. MORRELL, 1991). These authors also noted a remarkable similarity in appearance and texture between fungus-incited brown-rot decay and thermally-induced degradation of FRT wood. Each group of authors stated that this strength loss initially occurs before corresponding weight loss. This strength loss, as is also shown with ME 20, is not necessarily a function of weight loss (Fig. 3).

In addition, the studies by S. L. LEVAN et al. (1990) and J. E. WINANDY and J. J. MORRELL (1991) showed that significant strength loss occurs long before any appreciable degradation of glucose, which is associated with cellulose, is detected (Fig. 4). The limited glucose degraded was attributed to glucose associated in hemicellulose because the glucose was degraded at the same 1: 3 ratio as the glucomannan ratio of softwood hemicellulose. Thus, the remaining glucose, which the authors infer is associated with cellulose, was virtually unaffected. However, random cleavage of amorphous cellulose by acid may not produce detectable glucose (T. L. HIGHLEY, 1977).

E. SJOSTROM (1981) noted that hemicelluloses function to increase molecular packing density around the cellulose and act as the probable covalent link between lignin and carbohydrates. Thus, the removal of hemicelluloses through acid hydrolysis may increase the overall porosity of the wood. This increase in cell wall porosity might allow the diffusion of enzymes into the wood cell wall or low-molecular-weight polymers to the fungus. The depletion of hemicellulose may represent the transition point between incipient decay (acid-mediated depolymerization) and later stages of decay (enzyme-mediated utilization). Earlier studies showed that brown-rot fungi preferentially remove the hemicellulose of wood (T. L. HIGHLEY, 1987; T. K. KIRK and T. L. HIGHLEY, 1973). This is a critical issue since one of the current working theories about wood chemistry and strength is that the disruption of the highly branched hemicellulose structure, prior to cellulose or lignin degradation, accounts for significant strength loss without corresponding weight loss (J. E. WINANDY and R. M. ROWELL, 1984). Recent research on the effects of brown-rot decay on strength and chemical composition (J. E. WINANDY and J. J. MORRELL, 1991) and on the effects of acid fire-retardant-treated wood exposed at elevated temperature on strength and chemical composition (S. L. LEVAN et al., 1990) appears to confirm this theory.

The parallel studies with *P. placenta* ME 20 and MAD 698 (Table 4) provided further insight into the early decay mechanism of this fungus. Isolate ME 20 lacked the capacity to depolymerize cotton cellulose (Table 2). Figure 5 a provides ultrastructural evidence that highly organized cellulose bundles and/or microfibrils were intact and that packing material between these

bundles, composed mostly of hemicellulose, was modified or disrupted. The companion SEM of MAD 698 (Fig 5 b) presents the conventional view of brown-rotted wood as characterized by its amorphous appearance; when Figure 5 b is compared to Figure 6c, striking similarities are evident.

The FRT specimen in Figure 6b shows cellulose fibers in the S_2 layer, suggesting that the matrix was disrupted, and is comparable to Figure 5a of the ME 20 specimen. The amorphous nature of the S_2 layer and separation of the S_3 layer are seen clearly in both Figure 6c (MAD 698) and Figure 6d (FRT). However, we did not confirm whether cellulose bundles were depolymerized.

When the SEMs (Figs. 5 and 6) are viewed in concert with the fact that early strength loss is related to hemicellulose modification or disruption (Fig. 4). the figures apparently confirm the idea that biologically induced strength loss is initially a result of hemicellulose cleavage of side-chains and depolymerization of main-chains, followed by cellulose depolymerization.

We have demonstrated conclusively that oxalic acid can depolymerize cellulose at or near a variety of biologically realistic pH levels (1.5, 2.0, 2.5). This agrees closely with the data of M. SHIMADA et al. (1991). Thus, although hemicellulose breakdown appears to result, in part, in early strength loss, it is also probable that the acid effect accounts for the simultaneous rapid depolymerization of cellulose. We note that significant depolymerization of kraft pulp cellulose occurred with 1% and 5% oxalic acid to 39%, and 68% of untreated cellulose. respectively, after 4 weeks (M. SHIMADA et al., 1991). Our data show that oxalic acid at pH 1.6 (ca. 0.15 M) depolymerized cotton cellulose to 75% of its original condition within 7 days. T. L. HIGHLEY (1977) reported that oxidative systems may be involved in depolymerization of cellulose. Preliminary experiments indicated that the interaction of oxalic acid and cotton cellulose also results in generation of oxidized products, i.e., uranic and acetic acids, and increased carbonyls and carboxyls (unpublished results). Low levels of transition metals, like iron, may contribute to the formation of hydroxyl radicals in the presence of oxalic acid (E. ESPEJO and E. **AGOSIN,** 1991; C. J. SCHMIDT et al., 1981).

In the study reported here, as well as studies by K.-E. ERIKSSON (1990), F. GREEN et al. (1991), K. RUEL et al. (1990), and K. RUEL and J. P. JOSELEAU (1991), the sheath structure of wood-rotting fungi is noted as having potential significance as a medium through which decay enzymes must pass and eventually be delivered to woody substrates. Similarly, oxalic acid is also secreted by the fungus into the sheath environment. Although our evidence on the disposition of the oxalate anion is of a physical nature only, our SEMs suggest that oxalate (as calcium oxalate) always appears sequestered within the sheath matrix (unpublished observation).

The presence of an outer membranous sheath structure or pellicle may provide a semipermeable barrier to the oxalate anion, allowing the H_3O^+ ion to diffuse out and allowing Ca^{++} to diffuse in, thus forming calcium oxalate. Thus, the sheath may partition the environment between the fungal hyphae and substrates. Low pH in wood may also solubilize divalent cations, i.e., Fe, Ca, Cu. However, C. J. SCHMIDT et al. (1981) noted that an oxalate to iron ratio > 3:1 would immobilize iron, making it unavailable in the Fenton reaction. A. ENOKI (personal communication) stated that the one-electron (e^-) oxidation mechanism does not operate in oxalic acid solutions at low pH. Our data (unpublished) on the absence of detectable levels of hydrogen peroxide also suggest that the Fenton reaction is not an operative mechanism in our experiments.

The low pH reported here in wood (Fig. 1) was not detected previously. The pH levels we observed in situ suggest that low pH is responsible for early hydrolysis and depolymerization of carbohydrates and strength loss. Thus, initial stages of decay are apparently based simply on acid hydrolysis, in the absence of enzymatic activity, followed by increases in porosity caused by removal of hemicellulose, modification of lignin, and swelling of cellulose fibers (H. E. GRETHLEIN, 1985; B. PHILIPP et al., 1981 and G. UCAR 1990). Finally. a rise in pH may optimize enzymatic activity and penetration of these enzymes into the more porous wood. Alternatively, carbohydrate in the S_2 region is solubilized with subsequent diffusion of these carbohydrates through the S_3 layer to the sheath-enzyme complex on the S_3-lumen surface.

The complete mechanism of brown-rot decay of wood cannot be explained by oxalic acid production alone. The results of this report do not account for solubilization and utilization of cellulose beyond levelling-off degree of polymerization (LODP). The absence of reducing sugar groups or glucose following depolymerization of cotton cellulose by oxalic acid suggests the presence of additional biochemical steps prior to utilization by the fungus. Rapid decrease in pH may facilitate or activate other low-molecular-weight nonenzymatic agents or enzymes by solubilization and transport of divalent heavy metals, potentiation of additional acids (acetic, uranic, formic, citric, or butyric), and increased porosity of the wood.

5. Summary

The objective of this research was to determine the role of acid produced during early brown-rot decay by two isolates of *Postia placenta*, MAD 698 and ME 20. The results of direct pH measurement in wood blocks demonstrated a rapid decrease in pH to ca. 1.7 within 7 days by MAD 698. Estimation of oxalic acid production in vitro in woody substrates correlated with decreased pH. The results of in vitro treatment of southern pine blocks and cellulose with oxalic acid showed that acid can break down hemicellulose and depolymerize cellulose to a degree of polymerization of ca. 200 in 7

days. We conclude that low-molecular-weight acids are important in the initiation of brown-rot decay. The acid-mediated effect acts initially to break off hemicellulose side-chains, providing the fungus access to arabinose, galactose, and, to a lesser extent, other sugars. After initiation of acid-mediated hydrolysis of side-chains, acids begin to rapidly depolymerize and solubilize the hemicellulose backbones and amorphous cellulose, thus increasing the porosity of the wood structure to the hyphal sheath, decay enzymes, or other low-molecular-weight decay agents.

Zusammenfassung

Die Bedeutung van Oxalsäure bei beginnendem Braunfäulebefall

Ziel dieser Forschungsarbeit war, die Bedeutung der Säure zu bestimmen, die während eines beginnenden Braunfäulebefalls von den beiden Stämmen *Postia placenta* MAD 698 und ME 20 produziert wurde. Die Ergebnisse einer direkten pH-Bestimmung in Holzklötzchen zeigten innerhalb von 7 Tagen einen schnellen pH-Anstieg auf ca. 1,7 durch MAD 698. Eine Schätzung der Oxaisläreproduktion in vitro in Holzsubstrat korrelierte mit einer pH-Erniedrigung. Die Ergebnisse einer in vitro Behandlung von „southern pine"-Klötzchen und Cellulose mit Oxalägure zeigte, daβ die Säure die Hemicellulose abbauen und die Cellulose bis zu einem Polymerisationsgrad von ca. 200 innerhalb von 7 Tagen depolymerisieren kann. Wir schlieβen daraus, daβ niedermolekulare Säuren wichtig für den Beginn eines Braunfälebefalls sind. Die Säure bewirkt zuerst einen Abbau der Seitenketten der Hemicellulose und verschafft so dem Pilz Zugang zu Arabinose, Galactose und in geringerem Maβe zu anderen Zuckern. Nach der Hydrolyse der Seitenketten beginnt die Säure, die Hemicellulose-Struktur und die amorphe Cellulose schnell zu depolymerisieren und aufzulösen und so die Porosität der Holzstruktur für die Hyphen, die Abbauenzyme oder andere niedermolekulare Abbaustoffe zu erhöhen.

Résumé

L'importance de l'acide oxalique dans la détérioration initiale par la pourriture brune

On a étudié l'importance de l'acide oxalique produite dans la phase initiale d'une attaque par les deux souches des champignons de la pourriture brune *Postia placenta* MAD 698 et ME 20. Une détérmination directe du pH dans les blochets de bois a montré un abaissement rapide à environ 1,7 en 7 jours par le MAD 698. La production de l'acide oxalique dans des substrats de bois s'est révé1éé liéé à des valeurs pH plus faibles. Les résultats d'un traitement in vitro de pin de Douglas et de cellulose avec l'acide oxaliye a montré que l'acide est à même de décomposer la hémicellulose et de dépolymériser la cellulose à un degré de polymérisation d'environ 200 en 7 jours. Apparemment les acides de faible poids moléculaire jouent un rôle important dans les phases initiales de la décomposition du bois par les champignons de la pourriture brune. L'acide décompose d'abord les chaînes latérales de la hémicellulose permettant aux champignons l'accés à l'arabinose, la galactose et à d'autres sucres. Aprés l'hydrolyse des chaînes latérales l'acide commence à dépolymériser et a solubiliser rapidement la hémicellulose et la cellulose amorphe en augmentant la porosité de la structure du bois pour les hyphes, les enzymes de décomposition ou d'autres agents de décomposition de faible poids moléculaire.

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