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Brown-rot wood decay--insights gained from a low-decay isolate of *Postia placenta*

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ABSTRACT

Brown-rot Fungi represent one of the most economically important groups of wood decay micmorganisms. Some accounts estimate that as high as 80% of all in-service wood decay is caused by brown-rot Fungi. currant wood preservatives protect wood by means of broad spectrum biocides. Understanding the precise mechanism of brown-rot decay would permit development of targeted wood preservatives. A non-degradative strain of Postia placenta (ME-20) was studied to obtain information on defects in key decay mechanisms which prevent colonization. weight loss and hydrolysis of wood components, especially cellulose. The major defects of the monokaryon strain ME-20 are: 1) non-accumulation of oxalic acid on cellulose and wood; 2) nonfibrillar hyphal sheath: 3) Failure to translocate iron into wood in soil block tests; and 4) Inability to degrade pit membranes. The Failure to produce oxalic acid in the presence of pectin or cellulose or to degrade cellulose (wood or cotton) appear to be key characteristics of this nondegradative isolate.

INTRODUCTION

Brown-rot decay (BRD) is the most destructive and costly Form of decay of softwoods in service (1). The brown-rot mechanism can best be characterized by: i) acidic pH during colonization: ii) diffusion of low molecular weight, nonenzymatic decay agents (ie., Fe^{++}/H_2O_2) into the wood cell wall; iii) extensive depolymerization of polymeric polysaccharides and oxidation of lignin; and iv) measurable strength loss (modulus of rupture/modulus of elasticity: MOR/MOE) prior to significant weight loss (Fig. 1).

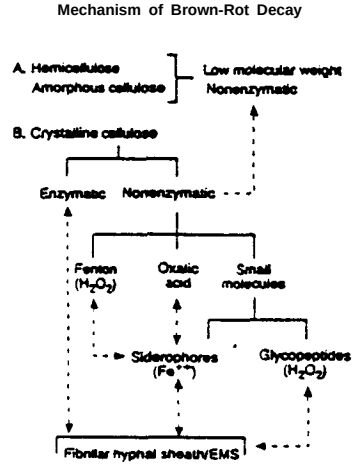

Figure 1 Proposed mechanisms of brown-rot decay. EMS-extracellular membrane structures (Ref. 78).

Brown-rot Fungi (BRF) produce a modest array of hemicellulases and cellulases with which to hydrolyze wood components. Hemicellulases are represented by xylanases, mannansses and polygalacturonases (2,3). Cellulases are generally limited to endoglucanases, and thus brown-rot Fungi lack the most important exoglucanase enzymes cellobiohydrolases (CBH) I and (CBH) II of Penicillium and Trichoderma species (4). One of the most interesting questions remaining about brown-rot decay is how brown-rot Fungi cause degradation throughout the wood cell wall early in decay

and solubilize the most recalcitrant hydrogen bond-ordered (crystalline) regions of cellulose with only endoglucanases and beta-glucosidases. It has been proposed that a nonenzymatic agent is involved because enzymes are too large to diffuse into the microstructure of wood (5).
Currently. proposed mechanisms For the nonenzymatic "exo- type" agent of brown-rot Fungi include (Fig. 1):

i) production of hydroxyl radicals by Fenton chemistry (6.7)

ii) acid-catalyzed hydrolysis by hydronium ion (8,9)

iii) reduction of iron by cellobiose dehydrogenase plus autoxidation (10, 11)

iv) one-electron oxidation (12)

v) production of low molecular weight chelators and iron-binding compounds (siderophores) (13)

Most evidence For Fenton chemistry during brown-rot decay is either anecdotal or circumstantial (14). There is not consensus as to which of these mechanisms is the real culprit, and the possibility exists For various genera of brown-rotters to have their own type of mechanism. However, certain key Factors seem common to all. All mechanisms seem to require an acidic wood environment, generally achieved by production of oxalic or other acids. All mechanisms seem also to rely on translocation and accumulation of iron (Fe^{+++})$_{++}$ into the wood Followed by conversion to Fe (15). One way to advance our understanding of the mechanism of brown-rot would be to examine the physiology of fungal cultures which have somehow lost their ability to degrade wood. It would be advantageous to have a number of mutant Fungal strains lacking in one or other of these metabolic or physiologic Features so that they could be characterized as to how this deficiency affects the strains capacity to decay wood or depolymerize cellulose. Rue1 and Eriksson (16) used a cellulase-less mutant of Sporotricum pulverulentum to study white-rot decay.

In 1961 a low decay monokaryotic culture of Postia placenta (ME-20) was isolated from floor planking in Pleasant Hill, California (17). The purpose of this chapter is to discuss the insights gained from this low decay isolate and how they might help solve the riddle of the mechanism of brown-rot decay and pin-point targeted protection methods.

FAILURE TO CAUSE WEIGHT LOSS IN WOOD

The most obvious defect, and thus the First to be observed. was the inability of isolate ME-20 to cause significant weight loss in wood when compared to other representive strains of P. placenta in standard soil block tests (18). While a standard decay strain, P. placenta MAD-698 caused about 60% weight loss in Southern pine blocks after 10-12 weeks, strain ME-20 only caused 5% - 9%, the lowest of all isolates of P. placenta tested (17). Thus, strain ME-20 behaves much like the molds Aspergillus niger and Trichoderma t-14 because it colonizes the wood but causes no appreciable weight loss or decay in soil-block tests (19). This defect is directly related to the inability of ME-20 to solubilise cellulose (see below).

IN VITRO CULTURAL CHARACTERISTICS

The colonial morphology of P. placenta ME-20 on agar has been shown to be quite different From many other brown-rot Basidiomycetes, especially other strains of P. placenta (Fig. 2). While degradative isolates are white and fluffy, producing many aria1 hyphae, ME-20 growth is thin, appressed and difficult to visualize (17). Light microscopy also revealed that ME-20 lacked clasp connections and was. thus monokaryotic. Pairing with other monokaryotic isolates of P. placenta produced dikaryons confirming its taxonomy (20). The radial growth rate of ME-20 on malt extract agar (MEA) was not different from MAD-698 (18).

In liquid culture, it was noted that the Filtrates of ME-20 were not as viscous as degradative strains such as MAD-698 (17). Further experimentation revealed that ME-20 Failed to produce extracellular polysaccharides in liquid culture. The extracellular polysaccharide of MAD-698 was primarily glucan composed of glucose residues (20). Glucose is the primary constituent of the non-fibrillar hyphal sheath in situ (Table 1).

One additional oddity of ME-20 is that it grows more readily on oxalate agar (pH 7.0) than do other BRF, a trait which closely resembles the physiology of white rot fungi (unpublished observation). This can be explained by the ability of ME-20 to raise the pH of culture media and grow at a more neutral pH than acid-loving BRF and not the enhanced utilization of oxalate as a carbon source.

PRODUCTION OF EXTRACELLULAR ENZYMES

In liquid culture, ME-20 produces much higher

Figure 2. Growth of four brown-rot fungi on 1% pectin agar. Note: Due to rapid in vitro growth the diameter of the colony of ME-20 is greater than the other three. It is just difficult to see in the appressed colony form.

rates of extracellular proteins than some of the degradative isolates. Elevated levels of the glycosidases, glucosidases and galactosidases, were measured. often 10-20 times higher than similar isolates capable of degrading wood (17). In the presence of cellobiose, ME-20 formed significantly higher amounts of extracellular proteins, however, carboxymethylcellulase (CMCase) was notably low. Strain ME-20 also produces endopolygalacturonase (Table 2). Even when the extracellular cellulose-degrading enzymes of ME-20 are extracted from wood. measurable endoglucanase activity in a viscosity reduction assay is detected (21). Three to four times more laminarinase was produced by ME-20 than MAD-698 (20). Thus, apparently, no degradative enzymes are absent in ME-20 when compared to MAD-698. ME-20 may raise the pH of its environment above the pH optima of its own enzymes. which for endopolygalacturonase is circa 4.5 (19).

FAILURE TO DEPOLYMERIZE COTTON CELLULOSE

Brown-rot fungi degrade cellulose in a manner that differs from that of other cellulolytic organisms. Shortly after colonizing wood, these fungi cause a rapid and extensive depolymerization of cellulose to the "limit" (length of cellulose crystallite) or crystalline degree of polymerization (DP) at low weight loss. Acid hydrolysis has a similar effect on cellulose. as do various strong oxidants. The cellulose that remains has an average DP of 150-200 and is more crystalline than nondepolymerized material because the cleavages occur in the amorphous. noncrystalline regions. The biochemical agent - or the system chat produces it - and causes this initial depolymerization is clearly a small diffusible agent because enzymes are too large to penetrate the wood cell wall to reach cellulose 22, 23). Furthermore. brown-rot fungi appear to degrade cellulose by a mechanism different from that of the synergistically acting systems involving exoglucanases present in white-rot fungi and Trichoderma (24). Uemura et al., (25) found that the cellulase system from brown-rot fungi gave a negative response towards antibodies to

Table 1. Comparison of Characteristics of MAD 698 and ME 20 isolates of *Postia placenta*

Isolate characteristic	ME-20	MAD-696
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
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 acid (pH - 2.0)	Yes	Yes

[a](Ref. 20)

[b]Depolymorization at 17 weeks

[c]pH $\leq$ 1.8

[d](Ref. 17)

[e](Ref. 37)

Table 2. Estimation of polygalacturonases activity, by reduction in viscosity of either sodium polypectate (20 g l) or pectin (20 g l) in relation to oxalic acid production, and weight lose in Southern yellow pine wood blocks (ref 3).

Fungus	Viscosity reduction ($10,000/t_{50}$)		oxa1ate (umol/mg dry wt)	weight loss (%)
	Sodium polypectate	Pectin		
None			0	0
P. placenta MAD-698	25.3	17.4	39	59
P. placenta ME-20	6.0	3.8	12	9
G. trabeum MAD-617	12.9	9.7	39	SO
S. incrassata MAD-563	7.4	2.3	48	50

Trichoderma CBH. suggesting the absence of homologous sequences and structures with the Trichoderma CBH. Attempts to identify the cellulose depolymerizing agent produced by brown-rot fungi have frustrated researchers for years. Almost 30 years ago. Cowling and Brown (23) recognized that even the smallest cellulases are too large to penetrate the pores of wood. Also, cellulases do not mimic the action of brown-rot fungi in generating cellulose crystallites (26, 27).

Degradation of cellulose-azure: Cellulose azure (Calbiochem. Sigma) is an acid-swollen cellulose preparation dyed with Remaxol brilliant blue R. With this method, dyed cellulose is layered onto an agar medium, inoculated with a fungus, the dyed products diffuse into the agar and as cellulose bonds are broken. The extent of color development indicates cellulolytic activity. Because it is swollen cellulose. it probably is not a true measure of the enzyme activity needed to solubilize crystalline cellulose. Micales and Highley (17) compared the ability of ME-20 to degrade cellulose asure with isolates of P. placenta having different wood decaying abilities. ME-20 produced a very weak reaction with cellulose azure, but so did certain isolates with good decay ability. Unlike the degradative isolates ME-20 could not degrade cellulose azure without the addition of 0.1% cellobiose. When the ability of ME-20 to degrade dyed cotton-based substrates (ie., amorphous, walseth, etc.) over agar is compared with that of P. placenta MAD-698, no differences were found (unpublished results).

Cotton cellulose in soil-block tests: When cotton cellulose is placed over an agar or soil medium so that it does not become saturated with water. brown-rot fungi that degrade wood are capable of degrading and utilizing cellulose (28). Weight loss of cellulose is the most accurate and reliable measure of exoglucanase activity (29). ME-20. however, does not degrade cotton cellulose exposed under these conditions. Addition of mannan or cellobiose to the cellulose did not promote utilization of cellulose by ME-20. although the DP's of the cellulose were lowered a bit (Table 3). At one week. only trace amounts of oxalic acid were produced by ME-20 on cellulose but with the co-substrates mannan or cellobiose. oxalic acid production was higher than that produced by MAD-698 on cellobiose. Interestingly, oxalic acid production by MS-20 plus the co-substrates decreased to only trace amounts by 12 weeks but oxalic acid continued to accumulate on the cellulose degraded by MAD-698.

Table 4 shows the results of a seven week soil block test to determine the effects of oxalic acid on depolymerization. weight loss, and crystallinity of cotton cellulose by P. placenta MAD-698 and ME-20. The control cotton without oxalic acid reached near limit of degree of polymerization (LODP) when cultured with MAD-698, but ME-20 had essentially no effect on DP or weight loss. However. the DP of both groups progressively decreased as the concentration of oxalic acid increased to 1.0N. Thus, it appears that the similar decrease in DP's of the two groups is

Table 3. Production of oxalic acid, weight loss and DP by P. placenta 698 and ME-20 on cellulose and cellulose plus a cosubstrate.[1]

		1 week			4 weeks			8 weeks			12 weeks		
P. placenta Isolate	Substrate	OA mg/l	WT Loss (%)	DP	OA mg/l	WT Loss (%)	DP	OA mg/l	WT Loss (%)	DP	OA mg/l	WT Loss (%)	DP
ME-20	Cellulose	2.7	0	988	1.5	0	1906	--	0	--	--	0	--
	Cellulose and 1% mannan	48.8	0	952	38.1	0	1498	20.9	0	1284	4.9	0	1420
	Cellulose and 1% cellobiose and minerals	132.2	0	700	3.8	0	1014	3.8	0	1058	6.9	0	1146
MAD-698	Cellulose	17.9	0	1460	22.9	0	414	64.0	26.7	250	72.0	44.7	230

[1]Cotton cellulose (50 mg) was soaked in the co-substrate and dried prior to placement in soil-block bottles. The mineral solution was that previously used by Highley (24).

Table 4. Effect of pretreatment of cotton cellulose samples (50 mg) with oxalic acid on degradation by MAD-698 and ME-20 after 7 weeks in soil-block tests. (ref. 30)

Osolate	Treatment	Weight loss (percent)	Oxalic acid (mmol)	Protein (ug/ml)	DP	CI[c] (percent)
MAD-698	1.0N OA	44	.234	1591	230[a]	81.97
"	0.1N	12	.287	1463	340	79.51
"	0.01N	4	.174	1241	450	78.15
"	0.001N	10	.160	985	814	78.46
"	H_2O	4	.252	1644	636	80.77
MAD-698	none	40	.210 (.219±.044)[b]	376	268[a] (80.1±1.5)[b]	81.48
ME-20	1.0N OA	40	.067	147	230[a]	77.67
"	0.1N	6	.026	104	360	79.67
"	0.01N	0	.025	98	700	81.85
"	0.001N	0	.021	91	1590	77.95
"	H_2O	4	.016	124	1740	76.63
ME-20	none	0	.042 (.032±.017)[b]	low	1976 (78.1±2.2)[b]	75.00
None	1.0N OA (4 weeks)				998	77.60
					586 (77.8±0.2)[b]	77.90
None	controls				1906	75.10
					2012 (76.3±1.2)[b]	77.52

[a]short fibers observed upon stirring in 2ml aqueous Triton-X 100

[b]mean±standard deviation of all treatments within a group (N=2)

[c]crystallinity index

due to oxalic acid pretreatment. Both fungal strains were able to colonize all cotton samples. This is demonstrated by protein measurements (Table 4). and enzyme assays (unpublished data). MAD-698 lowered the measurable oxalate near 0.200 mmo1 while the non-accumulator ME-20 decreased below 0.100 mmol. Each sample was performed in duplicate in order to compare washed and unwashed samples. Only the three samples (DP to 230) separated into short fibers upon washing. Comparisons of crystallinity indices revealed test percent crystallinity was increased slightly by all treatments (see Table 4). Thus, P. placenta MAD-698 has the capacity to lower the degree of polymerization. cause weight loss and produce "short fibers" on cotton. ME-20 fails to lower DP and produce short fibers without added oxalic acid (Table 4) (30).

Since oxalic acid can be synthesized commercially by the oxidation of sugars and sawdust with nitric acid (31) and since excess oxalic acid was measured after cellulose was degraded by the Fenton reaction in vitro (7). an interesting possiblity is raised that some fraction of oxalic acid produced during brown-rot decay is generated directly by the oxidation of glucose. Furthermore. Espejo and Agosin (32) proposed that when Gloeophyllum trabeum degraded cellulose. oxalic acid was converted concurrently to CO_2 and H_2O. Therefore, precise regulation of the degradation of oxalic acid by brown-rot fungi may contribute to cellulose DP and utilization (32). Also, unlike wood. cotton contains no metal elements to Facilitate Fenton-like

activity and ME-20 is deficient in metal ion transport (see below).

PRODUCTION AND ACCUMULATION OF OXALIC ACID

Brown-rot Fungi are, in general, accumulators of oxalic acid, the strongest of the organic acids (pKa=1.24)(33). Conversely. although white-rot Fungi produce oxalic acid, they are considered non-accumulators because of production of oxalate decarboxylase. Although certain brown-rotters like Gloeophyllum trabeum can be considered low accumulators when compared with P. placenta, Serpula incrassata, and Tyromyces palustrus, production of organic acids (acetic/formic) may compensate for less oxalate in certain genera. Although the precise role of oxalic acid is not totally defined, there is little doubt that acid-producing strains are more destructive than nonproducers (34, 35, 36).

Certain authors have described the production of oxalic acid as the key to the mechanism of brown-rot induced wood decay (36, 37). simply because it appears to be involved in many chemical processes simultaneously. First, oxalate is reported to be integrally involved. directly or indirectly. in the Formation of hydroxy radicals From H_2O_2 and iron. Oxalate may act as a reducing agent For conversion of Fe^{+++} to Fe^{++} required For Fenton chemistry to depolymerize polysaccharides (38, 7). Second, oxalic acid is a moderately strong iron chelator and the only chelator Found universally in brown-rot Fungi (10). Viikari and Ritschkoff (39) prevented brown-rot decay with both organic (EDTA) and inorganic (tripolyphosphate) iron chelators. Third. oxalic acid has also been implicated in pH reduction and direct acid catalyzed hydrolysis of the wood substrate (32, 37, 40). Fourth, oxalic acid is involved in chelation of other cations, i.e., Al^{+++}, Fe^{+++}, Ca^{++}, especially from the calcium pectate in pit membranes. compound middle lamellae (CML), ray parenchyma cells (36, 41, 42) and in the metal complexation of zinc (43). Production of oxalate would, therefore. enable the fungi to weaken the wood structure and increase the pore size to permit penetration by lignocellulolytic enzymes (44). Calcium oxalate frequently Forms crystals which can be readily visualized by scanning electron microscopy in fungi, wood. and soil. Oxalate may serve to sequester and detoxify excess calcium (44-48).

In our initial publication on production of oxalic acid by Postia placenta, we observed that while P. placenta MAD-698 lowered the pH of Southern-pine below pH 3.0 during the first week of colonization in soil block tests, ME-20 actually raised the pH more than one unit From approximately 4.0 to 5.0 (Fig. 3) (37). The lowered pH of MAD-698 was coincident with the production of oxalic acid over the first 7 days. after which there was a marked decline in the measuarable oxalic acid and **a** levelling off of the pH of the wood test blocks. Thus, we concluded that production of oxalic acid was a critical or key event during the initiation or colonization phase of brown-rot decay by P. placenta MAD-698. Low molecular weight acids appear to initiate the brown-rot process by catalyzing the hydrolysis of hemicellulose side chains, and ester groups (pectins) and providing the colonizing Fungus with a ready source of non-lignified carbohydrates. Without the capacity to accumulate oxalic acid and lower the pH of wood during colonization. ME-20 cannot hydrolyze the woody substrate and cause decay and weight loss.

However, as a Further complication of this situation. in vitro results are often at odds with in situ results (49). When Coniophora puteana was cultured in vitro with glucose as

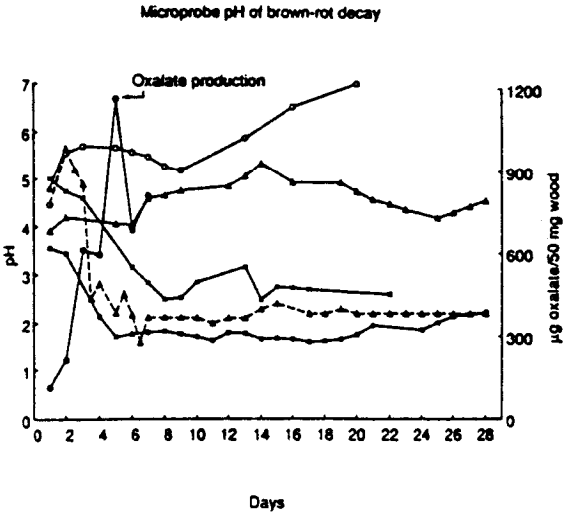

Figure 3 Continuous pH measurement of wood blocks by microprobe of brown-rot decay by Postia placenta isolates (ME-20/MAD-698) in southern pine. Oxalate production by MAD-698. Legend: 0 Control, ▲ME-20, X MAD-698 (run l), △ MAD-698 (run 2), ■ MAD-698(run 3) (Ref. 37).

a carbon source, the pH of liquid medium over 10 days dropped to 4.8 before increasing to 7.2, in spite of increasing production of oxalic acid (42).

ME-20 is not deficient in production of oxalic acid and in vitro often overproduces OA. ME-20 has been shown to lower the pH of MEA to pH 1.8 or below in 7 days similar to MAD-698 (37). Measureable oxalic acid has also been Found to be produced by ME-20 on a variety of substrates but not on xylan or pectin (19). In a recent study we compared the accumulation of oxalate in liquid culture For 7 brown-rot Fungi and 3 white-rot fungi (50). The minimal mediun was supplemented with sodium polypectate For a carbon source (see Table 5). ME-20 produced polygalacturonase and oxalic acid commesurate with MAD-698 Further emphasizing the disunity between its growth characteristics in vitro vs in situ. One conclusion which may be drawn from these experiments is that ME-20 has the capacity to produce oxalic acid in vitro especially at early time points but underproduces in situ (wood or cotton cellulose). The site of the precise defect has yet to be determined, but theoretically it could involve recognition of the woody substrate by the hyphal sheath or that the defective hyphal sheath of ME-20 prevents regulation of acidic pH. The second unique characteristic of ME-20 contributing to lack of oxalic acid is overproduction of oxalate decarboxylase, an enzyme which hydrolyzes oxalic acid (see below).

FAILURE TO TRANSPORT IRON AND OTHER METAL CATIONS INTO WOOD

Iron is an essential element For the growth. metabolism, and survival of the majority of cell types on earth. Although iron is a common element, it is rarely found in the Free form. Most organisms have developed an efficient chelation and/or regulated transport system, enabling the organism to get sufficient amounts of iron into the cell. Microorganisms use siderophores and corresponding membrane receptors For iron acquisition. Siderophores are defined as Ferric-iron specific ligands of low molecular weight (51). They are classified according to the chemical nature of the groups that bind Fe(III). The three general classes are hydroxamates, catecholates, and "other" non-conventional siderophores that do not contain hydroxamate or catecholate groups (52).

In a recent paper. Green et al., (53) demonstrated that brown-rot fungi translocate metallic cations into pine wood blocks during soil block tests (53). Essential elements (eg. Mn, Fs, Ca, Al) all increase during brown-rot. Table 6 demonstrates that although MAD-698 increased iron nearly 100-fold. no concommitant increase occurred in wood blocks colonized by strain ME-20. Although siderophores have been described For BRF (54). citric and oxalic acid production can also act as a metal chelator and Form yellow/orange halos on chrome azurol S (CAS) agar. Thus, oxalic acid is an effective chelator of iron and other metal cations and the failiure of ME-20 to transport iron into wood may result from its failure to accumulate OA in soil and lower the pH of wood in situ.

Thus, since iron has been shown to promote brown-rot decay (15), the Failure to translocate iron and other metal cations may also be a key defect in ME-20.

NON-FIBRILLAR HYPHAL SHEATH

A conspicuous ultrastructural Feature of wood decay Fungi is the presence of an extracellular matrix or hyphal sheath that covers the S3 layer of the wood cell wall during decay (55). The sheath appears to penetrate all wood cell wall layers and Forma the dynamic interface between the fungal hyphae and the wood (Fig. 4). Extracellular membraneous layers enveloping vegetative hyphae of fungi are a common morphological feature. Hyphal sheaths are postulated to provide several key Functions: e.g.. a) substrate attachment and recognition. b) nutrient reserve (glucan), c) reduce dessication, d) protection From toxic chemicals. e) Facilitate decay by storing or concentrating wood degrading agents. f) maintain a Favorable moisture/pH environment For enzymatic activity. and g) condition the substrate prior to enzymatic hydrolysis (56). Our laboratory has published extensively on the ultrastructure of the hyphal sheath of P. placenta MAD-698 and other brown-rot fungi (57, 58).

We have provided evidence For the existence of linear extracellular elements as structural components of the hyphal sheath of the brown-rot fungus P. placenta (58, 59). We have also located extracellular wood degrading enzymes weakly bound to the microfibrillar elements of the hyphal sheath using monoclonal antibodies to Beta-1,4-xylanase (59). ME-20's sheath has been observed by scanning electron microscopy (SEM) to exhibit an aberrant hyphal sheath structure on wood (37). Although it appears to penetrate the wood cell wall, cellulose bundles are not affected. P. placenta MAD-698 completely solubilizes the S2 layer of the wood cell wall but the cellulose bundles appear intact in pine colonized by

Table 5. survey of polygalacturonase activity, and oxalic acid production in liquid cultures of representative brown-rot fungi and gas permeabilities of Southern pine wood cores exposed to select fungi.

	Cup-plate Assay (mm)[1]	Viscosimetry (NR)[2]	Oxalic acid (umol/mg mycelium)	Gas Permeability (Darcys)
Brown-rot Isolates:				
Coniophora puteana (Schum.;Fr.) Karat. (Mad-515)	9	11.1	6.92	1.58
Postia placenta (Fr.) Lars. & Lomb. (Mad-698)	12	26.9	2.95	3.30
Gloephyllum trabeum (Pers.:Fr.) Murrill (Mad-617)	10	27.0	1.97	5.03
Postia placenta *(Fr.)* Lars. & Lomb. (ME-20)	12	24.9	1.57	2.03
Antrodia Carbonica (Overh.) Gilbn. & Ryv. (HHB-5104)	8	10.1	11.52	2.06
Formitopsis paulstris (Berk. & Curt.) Gilb & Ryv.(L-15755)	10	8.6	46.89	4.08
Formitopsis pinicola (Fr.) Karst. (105877)	10	6.4	47.71	4.25
White-rot Isolates:				
Trametes versicolor (L.:Fr.) Pil. (Mad-697)	9	0	0	13.63
Irpex lacteus (Fr.:Fr.) Fr. (HHB-7328)	11	4.8	0	18.76
Phanerochaete chrysosporium Burds. (ME-461)	4	9.4	0	22.18
Mold Isolates:				
Aspergillus niger	2	5.6	*NO*	ND
Trichoderma barzianum ATCC 20476	10	0	ND	ND
POS. Control[3]	13	22.6		0.64

[1]Well diameter (5mm) was subtracted from each total precipitin measurement.

[2]Viscometric data are expressed as $10,000/t_{50}$ per ml enzyme soultion, where t_{50} equals the time for relative viscosity of the solution to be reduced by

[3]Positive control equals 0.9 units pectinase activity/ml.

ND=not determined (Ref. 50)

Table 6. Elemental analysis of sound (CONSYP) and brown-rotted Southern pine by ICP spectroscopy.

	%	PPM										
SAMPLE*	Total N	P	K	Ca	Mg	S	Fe	Mn	Zn	Cu	Al	Na
CONSYP	.03/.03	18/20	246/276	548/584	133/139	27/33	1/1	120/129	6/l0	2/2	29/31	68/130
MAD-698	.13/.17	65/97	178/180	1612/1860	514/573	128/136	62/106	257/276	l/lO	5/10	40/46	73/109
ME-20	.8/.11	17/22	80/62	1076/1162	383/411	84/80	1/1	180/182	5/10	2/2	28/35	48/72

* Duplicate wood samples
(ref Green IBB 1997)

Figure 4 Scanning electron micrographs of decayed southern pine and hyphal sheaths (sh) for Postia placenta isolates MS-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 solubilized by ME-20-S_3 layer visible on left; (b) amorphous S2 decayed by MAD-698-hyphae (h). S_2 and S_3 layers, and compound middle lamella (cml) visible; (c) smooth 10000. Scale bar = 1 um (Ref. 37).

ME-20 (Fig. 4). The aberrant hyphal sheath of ME-20 may be related in some way to the inability to solubilize cellulose into glucose and produce glucan.

FAILS TO DEGRADE PIT MEMBERS DURING COLONIZATION

Colonization of wood by fungal hyphae is an early and important event in the sequence of wood decay. It can progress either by pit hydrolysis, bore holes or both (60). Recently. we reported the in vitro induction of polygalacturonase (Pa) and oxalic acid by pectin in brown-rot fungi (3). Studies of pectin degrading enzymes in wood decay fungi are Scarce due to the relatively low content of pectin in wood (trace to 4%). However, we concluded that the pectin is contained within certain key anatomical wood locations which may add to it's importance during colonization and incipient decay by decay fungi. Pectin is localized mainly in the primary wall. middle lamella in the cell corners, ray parenchyma cells and tori of the bordered pit membranes (Fig. 5) (61, 62). Wood presents a relatively inhospitable surface for fungi to colonize due to the lignification of many surfaces like the S3 layer of the wood cell wall (see Fig. 5). Ray parenchyma cells and the tori of bordered pit membranes may represent areas of non-lignified carbohydrates available to facilitate the spread of hyphae throughout the woody substrate and provide open avenues for colonization not requiring degradation of the entire wood cell wall to gain access to the next cell. Brown-rot fungi may conserve the functions of oxalic acid shown to be keys to invasion by plant pathogenic fungi: i) direct disintegration of pectic substances, especially weak ester linkages, ii) synergistic action of oxalic acid and PC and iii) lowering the pH and chelating the calcium bound to the pectin (63). Tschernitz and Sachs (64) provided the first experimental evidence that oxalic acid is required for pit hydrolysis. Without the accumulated oxalic acid, ME-20 fails to dissolve pits, chelate Ca^{++}, or increase wood permeability (3), and thus fails to open up the porosity of wood

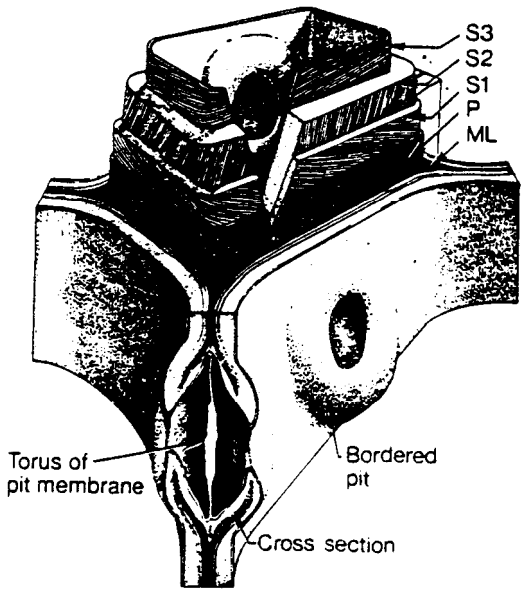

Figure 5 Bordered pit structure and its relationship to the layers of the wood cell wall of tracheids. Note that the bordered pit membrane is not aspirated. Cell wall layers: Middle lamella (ML). primary wall (P), Sl, S2, S3 (Ref. 3).

Table 7. Effect of brown-rot decay on lonqitudinal gas permeability and weight loss of Douglas-fir sapwood cores (6.50mm).

Day	7	12	12
Fungus (isolate)			percent (weight loss)
P. placenta (MAD-698)	4.760[1]	10.890	15%
P. placenta (ME-20)	0.243	0.197	0%
G. trabeum (MAD-617)	1.419	8.970	4%
S. incrassata (MAD-563)	1.069	9.240	36%
control[2]	0.0791	0.276/0.474	0%

[1]Dazcys (Milota 1994) [2]pits aspirated [3]reference (3, 19)

(36).

was permeability of 6.4mm Douglas fir cores following incubation with brown-rot fungi is shown in Table 7. Three of four brown-rot fungi increased gas permeability during incipient decay. When compared to the other three fungi, P. placenta ME-20 does not lower pH, cause wood weight loss, or accumulate oxalic acid. The latter is probably due to overproduction of oxalate decarboxylase, thought until recently to only be produced by white-rot fungi (65). Even with production of polygalacturonase, permeability of wood does not increase and pits are not degraded. most likely because of low oxalic acid production (Table 2). This inability of ME-20 to degrade pits has also been observed by TEM (Fig. 6) (3). Note in figure 6(b) that the pit torus and margo remains intact in ME-20 colonized pine.

It should also be noted that the inability of ME-20 to penetrate the pit membranes may also be related to ME-20's inability to utilize cellulose. The margo of the pit membrane is comprised of cellulose embedded in hemicellulose (66). Cellulases are very important in pit membrane hydrolysis. In addition, oxalic acid produced locally during pectin hydrolysis of pit tori may damage cellulose microfibers in pit margos (67).

BIOCONTROL OF WOOD DECAY BY ME-20

A variety of methods are available to inhibit the growth of wood decay fungi (chemical preservatives, biocides, metabolic inhibitors. fungicides) but they all depend on the manipulation of the substrate in such a way as to prevent degradation of the wood cell wall.

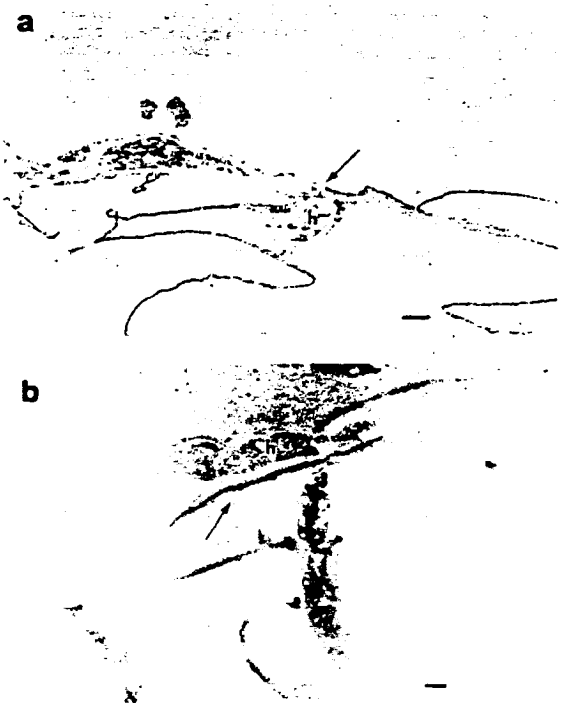

Figure 6 Transmission electron micrographs of bordered pits of southern yellow pine in soil block test (a) 10 days post-inoculation with (a) P. placenta MAD-698, showing hyphae penetrating the bordered pit membrane (arrow), and (b) 31 days post-inoculation with P. placenta ME-20, showing hyphae on both sides of the pit membrane but no penetration. Bar = 1 um (Ref. 31.

In the natural environment. an additional Factor must be taken into account, competition From other Fungi and bacteria.
Protection of timber by pretreatment with other living microorganisms (i.e., Trichoderma) has been shown to effectively limit the growth and the decay of wood degrading Basidiomycetes (68).

Since ME-20 can colonize wood without producing strength or weight loss, we decided that the isolate should be looked at for it's biocontrol potential. Initially, we compared MAD-698 and ME-20 for their abilities to produce metabolites that were inhibitory to decay Fungi on a malt-agar medium. The metabolites produced by ME-20 were much more inhibitory to the decay Fungi than those produced by MAD-698 (Table 8). Next, we looked at the ability of ME-20 to prevent decay in wood blocks. ME-20 completely inhibited decay by MAD-698 but only slightly reduced decay by the other Fungi (Table 9). When ME-20 was killed, it lost its ability to prevent decay by MAD-698. This suggests that ME-20 does not produce residual metabolites in wood inhibitory to MAD-698. Thus, these results indicate that although ME-20 produces Fungal inhibitory metabolites. ME-20 probably has little potential as a successful biocontrol agent. It is interesting, however, that the degradative MAD-698 isolate does not produce fungal inhibitory metabolites. Possibly, the production of fungal inhibitory materials by ME-20 plays a role in its inability to decay wood.

OVERPRODUCTION OF OXALATE DECARBOXYLASE

Shimazano (69) First described the enzyme. oxalate decarboxylase, and associated it specifically with the inability to accumulate oxalic acid in white-rot fungi. In 1964, enzymatic oxalate decarboxylation described in Aspergillus niger (70). Oxalate decarboxylase (ODC) hydrolyses oxalic acid to Formic acid and carbon dioxide. Recently, Micales (71) was the first to report that oxalate decarboxylase can also be isolated from both the normal (MAD-698) and the non-degradative (ME-20) isolates of P. placenta. Micales (71) demonstrated that the optimal pH of ODC is between 1.75 and 2.20 which is close to the "optimal" pH of brown-rotted wood (3). When grown in vitro in liguid culture, ME-20 accumulates oxalic acid for the first 10-12 days and then it disappears. One hypothesis. chat ODC stays cell bound, perhaps to the hyphal sheath of P. placenta MAD-698, but is released extracellularly by ME-20, may be an oversimplification. At some of the test intervals (11d). ME-20 makes as much as 50 times more ODC than MAD-698, thus ME-20 hydrolyze all available oxalic acid while MAD-698 may only hydrolyzes the oxalic acid in its immediate microenvironment (Table 4, 72).

Table 8. Inhibition of fungal growth by metabolites produced by P. placenta (ME20) or (698) in the cellophane test over malt agar.

Wood Decay Fungus/Isolate	Inhhibition of fungal growth (percent) ME-20 on Cellophane	MAD-698 on Cellophane
P. placenta (698)	34.4	4.3
P. placenta (ME-20)	13.7	0.0
S. incrassata (563)	72.3	6.6
G. trabeum (617)	78.1	18.8
C. versicolor (697)	100	35.6

[1]Cellophane was placed over 2% malt-agar medium in Petri plates, inoculated with ME-20 or 698, and the cellophane removed when fungal growth covered the cellophane. Plates were then inoculated in the center with a decay fungus and periodic growth measurements made.

Table 9. Biocontrol of brown-rot and white-rot fungi by P. placenta ME-20 on wood.

Decay Fungus/Isolate	Height loss[1] produced by wood-rotting Basidiomycetes (percent)		
	Blocks pretreated with M-20[2]		
	Living ME-20	Killed ME-20	Control
BROWN-ROT			
P. placenta (698)	0.0	37.8	40.5
S. incrassata (563)	41.1	51.7	48.5
G. trabeum (617)	31.8	37.5	39.4
WHITE-ROT			
C. versicolor	47.3	56.8	60.6

[1]Exposed 10 weeks in soil-block test. Southern pine blocks were used for the brown-rotters and sueetgum for the white-rot funqus.

[2]Blocks exposed to ME-20 for 6 weeks in the soil-block test. NO weight loss in blocks. Half of the ME-20 created blocks were gas-sterilized to kill ME-20 before exposure to the decay fungi.

Table 10. Failure of P. placenta (ME-20) to decay pine previously exposed to P. placenta (698).

Days exposed to 698	Weight loss produced by 698 prior to exposure to ME-20 and 698.	Weight loss produced in soil-block tests after 10 weeks.	
		ME-20	698
7	0	1.7	66.5
10	4	3.6	70.0
14	10	5.1	66.0

[1]Cellulose (cotton) also tested as above but ME-20 was not able to produce weight loss in any of the cellulose previously exposed to 698, while 698 able to produce weight losses of 65-68 percent.

Due to the observations that ME-20 has a nonfibrillar hyphal sheath low in glucan. this defect may result in the "release" of excess ODC into the surrounding environment by ME-20 which hydrolyzes all available oxalic acid and aborts the decay process. However. recent

experiments suggest that even in ME-20. ODC is bound to the hyphal sheath (72). Nevertheless, due to overproduction of ODC and hydrolysis of oxalic acid. the pH of the wood is raised and the synergism between oxalic acid and enzymes like polygalacturonase is minimized.

DECAY OF WOOD OR CELLULOSE BY ME-20 FOLLOWING EXPOSURE TO MAD-698

Possibly the degradative MAD-698 isolate could predispose wood or cellulose to attack by ME-20 and, thereby, provide clues as to why ME-20 is incapable of decaying wood or cellulose. For example, exposure to MAD-698 might render substrates accessible to ME-20 and permit subsequent decay. We exposed cotton cellulose and Southern pine blocks to MAD-698 For 7, 10, and 14 days in soil-block tests and then propylene oxide-sterilized them before exposure to MAD-698 in soil-block tests (Table 10). ME-20 was unable to cause weight loss in any of the cellulose previously exposed to MAD-698, while MAD-698 produced weight losses of 65-68 percent. ME-20 produced small weight losses in wood previsouly exposed to MAD-698 (1.7 - 5.2%) while MAD-698 caused 66-70% weight loss in previously exposed pine blocks. Thus, MAD-698 was unable to predispose cellulose or wood to attack by ME-20.

CONCLUSIONS

Shortly after colonizing wood. brown-rot fungi cause a sharp and rapid reduction in the degree of polymerization (DP) of holocellulose with concommitant strength loss without removing lignin (22). To accomplish this. low molecular weight. BRD agents must penetrate the microstructure of the wood and diffuse into the wood cell wall. The porosity of sound wood is too small to permit diffusion of enzymes (73, 74). It is clear that ME-20 cannot effectively generate these low molecular weight decay agents.

Many anomalies and enigmas still exist with regard to strain ME-20: e.g., First, the resilts of *in vitro* experiments shedding light on wood decay is not very likely. In many *in vitro* tests. ME-20 outperforms MAD-698. but, not in causing weight loss of wood or cotton *in situ*. Second. the 5% weight loss due to colonization by ME-20 is not totally understood. Since hemicellulose is removed very early during the decay process (18, 37) and the hyphal sheath appears to solubilize the matrix surrounding cellulose bundles (Fig. 2), it is possible that utilization of hemicellulose supports the growth of ME-20 *in situ*. Third. how does the ODC of ME-20 hydrolyze all the oxalic acid and raise the pH when the optimal pH of the enzyme is 1.8 - 2.0? (65, 71). Fourth, we are unable to pinpoint the precise genetic defect which cripples strain ME-20. Isolates such as ME-20 with altered physiological activities could be valuable For the identification of genes which code for the enzymes involved in the complex process of wood decay. Variation at the DNA or protein level could be used to potentially identify these genes. It would be interesting to compare the genetic differences of the *P. placenta* isolates using such methods as DNA variation. SDS profile and the polymerase chain reaction (PCR). Fifth. it is reported that Fenton chemistry is inhibited by excess oxalic acid. In spite of the fact that ME-20 hydrolyzes all OA, how does the brown-rot fungus, *Fomitopsis palustris* (L-15755) circumvent very high oxalate accumulation (75, 14)? Sixth, how does ME-20 lower the pH of malt agar to 1.8 but cannot do the same with wood (37)?

The key observation that ME-20 is unable to hydrolyze pit membranes, fully colonize the wood and increase wood permeability has led to the testing of a new generation of wood preservatives targeted specifically at pectin to prevent pit hydrolysis and the cascade of events which follows colonization. our laboratory has been successful at preventing wood decay with calcium precipitating agents like N,N-napthaloylhydroxylamine (NHA) (77, 76). Studies of ME-20 have led to important insights into the mechanism of brown-rot decay. Experiments are underway to further explore and better characterize the defects of this interesting. low decay isolate of *P. placenta*.

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