

# Production of Polygalacturonase and Increase of Longitudinal Gas Permeability in Southern Pine by Brown-Rot and White-Rot Fungi

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## Keywords

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White-rot  
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Pit membrane  
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Oxalic acid

## Summary

Hydrolysis of bordered and pinoid pits may be a key event during colonization of wood by decay fungi. Although pits are numerous, studies of pectin-hydrolyzing enzymes in wood decay fungi are scarce, probably because of the relatively low content (less than 4 %) of pectin in wood and because of the primary focus on understanding the degradation of lignified components. Endopolygalacturonase (endo-PG) activity was estimated by cup-plate assay and viscosity reduction of pectin from liquid cultures of fifteen brown-rot and eight white-rot basidiomycetous fungi using sodium polypectate as the carbon source. Oxalic acid was estimated in liquid culture and related to mycelial weight of each fungus. Changes in longitudinal gas permeability of southern pine cores exposed to selected decay fungi in liquid culture were measured to determine the extent of hydrolysis of bordered pits. Twelve of fifteen brown-rot and six of eight white-rot fungi tested were positive for at least one of the polygalacturonase test methods. Accumulation of oxalic acid was detected in thirteen of fifteen brown-rot isolates and none of the white-rot fungi tested. Gas permeability of pine cores increased approximately fourfold among brown-rot fungi tested and eighteenfold among white-rot fungi tested. Scanning electron microscopy revealed bordered pit membrane hydrolysis in cores colonized by white-rot fungi, but only torus damage, weakening and tearing of the pit membranes, was observed in cores exposed to brown-rot fungi. We conclude that both brown- and white-rot decay fungi have the enzymatic capacity to hydrolyze pectin, damage bordered pit membranes, and increase wood permeability during colonization and incipient decay.

## Introduction

During colonization of wood by decay basidiomycetes, the fungal hyphae need to ramify through the wood and gain access to easily metabolizable forms of carbon in a low nitrogen environment (Cowling 1961). Hydrolysis of wood pectin from ray parenchyma cells and the tori of pit membranes has been hypothesized as a necessary step in the colonization of wood by brown-rot fungi (Daniel *et al.* 1994; Green and Highley 1997). *Trichoderma* sp. have been shown to increase wood permeability by degrading pit membranes (Sharma and Kumar 1979). Enzymatic degradation of pit membranes has also been observed in white-rot fungi (Tsuneda *et al.* 1987). Shanley *et al.* (1993) characterized endopolygalacturonase (endo-PG) from the white-rot fungus *Phanerochaete chrysosporium*. Degradation of pit membranes by microbial enzymes is well documented (Green *et al.* 1996). Green *et al.* (1995a) showed that the pit membranes of southern pine (*Pinus* spp.) were degraded by day 14 in soil-block tests by three brown-rot fungi. This mechanism of invasion was further supported by the inhibition of decay by the selective precipitation of calcium by N'-N-naphthaloylhydroxylamine (NHA) (Green *et al.* 1997a,b). Most wood calcium is bound to pectin, which

must be chelated prior to enzymatic hydrolysis, often by oxalic acid, which acts synergistically with endo-PG (Magro *et al.* 1984; Bailey and Reeve 1994). Since white-rot fungi were also inhibited by NHA to the same degree as brown-rot fungi, the question arose whether other wood decay fungi also hydrolyze pectin as a readily available source of nonlignified carbohydrate during colonization and early stages of decay. Therefore, one approach for inhibiting fungal colonization of wood would be to prevent pectin hydrolysis.

This paper is the first survey of pectin-degrading enzymes by representative cultures of brown- and white-rot fungi. The objectives of this study were to estimate polygalacturonase activity and oxalate production in brown- and white-rot fungi and to measure the gas permeability of southern pine cores following incubation *in vitro* with decay fungi. We conclude that both brown-rot and white-rot fungi are suitably equipped with enzymes and chelators capable of depolymerizing wood pectin and hydrolyzing pit membranes. Based upon estimated increases in gas permeability, white-rot fungi have the advantage (*in vitro*) of producing a complete array of exo- and endo-cellulases capable of removing the margo of pit membranes as well as the pectin-rich torus (Highley 1974, 1975, 1977).

## Materials and Methods

### Fungal isolates and growth conditions

Brown- and white-rot fungi used in this study are listed in Table 1. Cultures of each isolate were maintained on 2 % malt agar. Liquid cultures were grown on Bailey's minimal medium (Highley 1973) supplemented with 0.5 % sodium polypectate. Cultures were incubated for 9 d at 27 °C at 200 rpm on a rotating table.

### Oxalate determination and enzyme assays

Oxalate was estimated using a microadaptation of a commercial enzymatic oxalate assay (Sigma Chemical Co., St. Louis, MO, U.S.A.). Polygalacturonase was determined by two methods, a cup-plate assay (Dingle *et al.* 1953) and the reduction in viscosity of citrus pectin (Eastman Organic Chemicals, Rochester, New York, U.S.A.) measured in a Cannon-Fenske viscometer (Highley 1973; Clausen and Green 1996). Cup-plate data are direct measurements of the diameter of the zone of clearing minus the diameter of the well compared with a commercial standard pectinase (Sigma Chemical Co.). Viscometric data are expressed as

$10,000/t_{50}$  per ml enzyme solution, where  $t_{50}$  equals the time (s) for the relative viscosity of the solution to be reduced by 50 % at 28 °C.

### Gas permeability

Southern pine cores (64-mm diam.) were bored from green sapwood, steam sterilized, and incubated in 100-ml liquid culture as described in growth conditions. Cultures were inoculated with selected fungi from Table 1. After 3 weeks of exposure to the fungus, the cores to be tested for gas permeability ( $n = 3$ ) were removed and oven-dried at 72 °C (Tschernitz and Sachs 1973). Permeabilities were measured and calculated after the method of Milota *et al.* (1995).

### Electron microscopy

Pine cores to be examined by scanning electron microscopy (SEM) were dried at 40 °C, split in the radial plane, mounted on stubs, and gold-coated using a Denton sputter coater Desk-1 (Cherry Hills, NJ, U.S.A.). Samples were examined and photographed using a Hitachi S530 (Tokyo, Japan) or JEOL 840 (Peabody, MA, U.S.A.) scanning electron microscope at 5 and 15 kV.

**Table 1.** Survey of polygalacturonase activity and oxalic acid production in liquid cultures of representative brown- and white-rot fungi and gas permeabilities of southern pine wood cores exposed to select fungi

	Cup-plate assay (mm) <sup>a</sup>	Viscosimetry (VR <sub>50</sub> ) <sup>b</sup>	Oxalic acid (µM/mg mycelium)	Gas permeability <sup>c</sup> (Darcy's)
<b>Brown-rot isolates</b>				
<i>Coniophora puteana</i> (Schum.:Fr.) Karst. (Mad-515)	9	11.1	0.69	1.58
<i>Postia placenta</i> (Fr.) Lars. & Lomb. (Mad-698)	12	26.9	0.30	3.30
<i>Neolentinus lepideus</i> (Fr.:Fr.) Redhead et Ginns (Mad-534)	6	0	0	ND <sup>d</sup>
<i>Gloeophyllum trabeum</i> (Pers.:Fr.) Murrill (Mad-617)	10	27.0	0.20	5.03
<i>Serpula incrassata</i> (Berk. & Curt.) Murrill (Mad-563)	4	0	1.17	ND
<i>Antrodia xantha</i> (Fr.:Fr.) Ryv. (Mad 5096-35)	7	0	0.79	ND
<i>Laetiporus sulphureus</i> (Fr.) Murrill (W) Sh-27-R	0	0	0.36	ND
<i>Laetiporus sulphureus</i> (Fr.) Murrill (E) Boat-206	2	13.0	3.90	ND
<i>Postia placenta</i> (Fr.) Lars. & Lomb. (ME-20)	12	24.9	0.16	2.03
<i>Antrodia carbonica</i> (Overh.) Gilbn. & Ryv. (HHB-5104)	8	10.1	1.15	2.86
<i>Antrodia serialis</i> (Fr.:Fr.) Donk (FP-104443)	0	0	0	ND
<i>Antrodia vaillantii</i> (DC.:Fr.) Ryv. (FP-90877)	0	0	2.51	ND
<i>Fomitopsis palustris</i> (Berk. & Curt.) Gilb & Ryv. (L-15755)	10	8.6	4.69	4.08
<i>Phaseolus schweinitzii</i> (Fr.) Pat. (105389)	0	8.1	0.10	ND
<i>Fomitopsis pinicola</i> (Fr.) Karst. (105877)	10	6.4	4.77	4.25
<b>White-rot isolates</b>				
<i>Trametes versicolor</i> (L.:Fr.) Pil. (Mad-697)	9	0	0	13.63
<i>Phlebia brevispora</i> Nakas. (HHB-7030)	0	0	0	ND
<i>Irpex lacteus</i> (Fr.:Fr.) Fr. (HHB-7328)	11	4.8	0	18.76
<i>Bjerkandera adusta</i> (Fr.) Karst. (L-15359)	0	7.4	0	ND
<i>Phanerochaete chrysosporium</i> Burds. (ME-461)	4	9.4	0	22.18
<i>Phanerochaete chrysosporium</i> Burds. (ME-446)	6	7.1	0	ND
<i>Ganoderma applanatum</i> (Pers.) Pat. (HHB-7823-S)	8	8.3	0	ND
<i>Xylobolus frustulatus</i> (Pers.:Fr.) Boid. (FP-24059)	0	0	0	ND
<b>Mold isolates</b>				
<i>Aspergillus niger</i> van Tieghem	2	5.6	ND	ND
<i>Trichoderma harzianum</i> Rifai (ATCC 20476)	10	0	ND	ND
Positive control <sup>e</sup>	13	22.6		0.64

<sup>a</sup>Well diameter (5 mm) was subtracted from each total precipitin measurement.

<sup>b</sup>Viscometric data are expressed as  $10,000/t_{50}$  per mL enzyme solution, where  $t_{50}$  equals the time for relative viscosity of the solution to be reduced by 50 % at 28 °C. VR<sub>50</sub> is 50 % viscosity reduction.

<sup>c</sup> $n = 3$ .

<sup>d</sup>ND, not determined.

<sup>e</sup>Positive control equals 0.9 units pectinase activity per milliliter.

## Results

Table 1 shows comparative results for polygalacturonase activities in fifteen brown-rot fungi and eight white-rot fungi by two methods of analysis. Eight of the fifteen brown-rotters were positive for both methods of evaluation. Three brown-rotters, *Laetiporus sulphureus* and two *Antrodia* sp., were negative for polygalacturonase activity under the conditions tested.

Six of eight white-rot fungi tested were positive for one or both methods of evaluation for polygalacturonase activity. Two organisms, *Phlebia brevispora* and *Xylobolus frustulatus*, were negative for polygalacturonase activity.

Table 1 also shows oxalic acid results for liquid cultures of all isolates. The two species of *Fomitopsis* and one isolate of *Laetiporus sulphureus* (Boat 206) demonstrated the highest concentration of oxalic acid accumulation per milligram of mycelium. None of the white-rot fungi accumulated measurable oxalic acid, and only two brown-rot fungi, *Neolentinus lepideus* and *Antrodia serialis*, did not accumulate measurable oxalic acid.

The fungal isolates with highest polygalacturonase activity were tested for their ability to alter the longitudinal gas permeability of southern pine cores. White-rot fungi, *T. versicolor*, *I. lacteus*, and *P. chrysosporium*, were able to increase gas permeability in vitro to a greater degree (13.63

to 22.18 darcy's) than the seven brown-rot fungi, which ranged from 1.58 to 5.03 darcy's (Table 1).

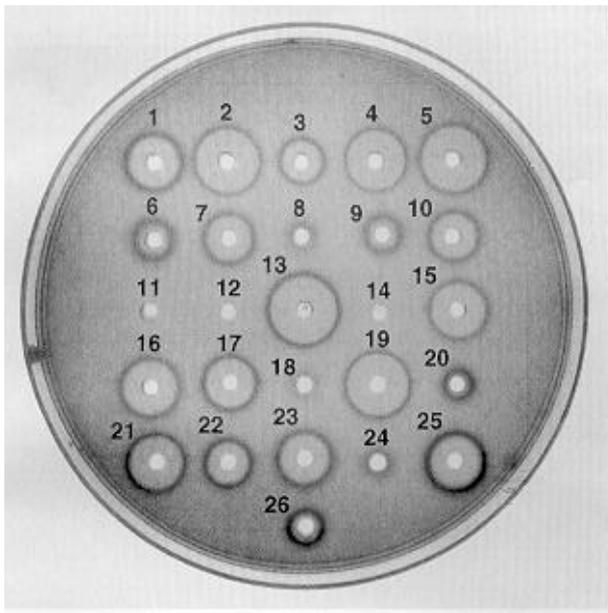
Figure 1 shows the cup-plate assay for polygalacturonase. Eighteen of twenty-five fungi tested were positive for the cup-plate assay (2- to 12-mm-diameter reactions). The control value of commercial pectinase (0.9 units activity/ml) was 13 mm.

Figure 2 shows scanning electron micrographs of bordered pit membrane damage of two brown-rot fungi (A and B) and two white-rot fungi (C and D). All pit membranes were aspirated. The majority of brown-rot exposed pit membranes examined were visible but partially eroded or torn, while the majority of white-rotted pit membranes examined were gone. In Figure 3, a pit membrane from a control pine core (A) is compared with a pit membrane that had been exposed to *G. trabeum* (B). The torus of the treated pine core has been eroded, leaving the cellulose microfibrils of the margo exposed.

## Discussion and Conclusion

This survey supports the hypothesis that most brown- and white-rot fungi have the enzymatic capacity to hydrolyze the pectin in pit membranes during incipient decay, which facilitates colonization. These results support observations by Cowling (1961) and Wilcox (1968). They demonstrated that during early stages of brown-rot decay by *Postia* sp., the hyphae ramified through the entire wood block prior to 5 % weight loss, mostly by penetration of pinoid and bordered pits. Likewise, Tsuneda *et al.* (1987) observed enzymatic degradation of pit membranes by the white-rot fungus, *Lentinus edodes*, in oak (*Quercus* spp.). Bore holes also provide a potential route of hyphal attack from cell to cell during fungal colonization of wood, but hydrolyzing the nonlignified pit membranes should provide more rapid and efficient access to metabolizable substrates (Doi and Nishimoto 1985; Kuo *et al.* 1988). It has been clearly established that two synergistic events must occur for the degradation of pectin in the torus of bordered pit membranes (Bateman and Beer 1965; Tschernitz 1973; Tschernitz and Sachs 1973). First, the fungus must chelate the calcium sequestered between the polygalacturonic acid chains. This is most likely accomplished by chelation with oxalic acid. Second, the fungus must produce an endopolygalacturonase to break the alpha 1-4 linkages in the polygalacturonic acid chains (Bateman and Beer 1965). Concurrent with pectin degradation, the interlacing cellulose microfibrils of the pit margo may also be hydrolyzed by exo- and endo-cellulases or their functional equivalent in the case of brown-rot.

It has been reported that oxalic acid is involved with pH reduction and acid-catalyzed hydrolysis of the wood substrate (Green *et al.* 1991, 1992, 1994; Espejo and Agosin 1991; Shimada *et al.* 1991). Oxalic acid also chelates the pectin-bound calcium in pit membranes, the compound middle lamellae, and ray parenchyma cells, and it is observed as calcium oxalate crystals by SEM (Bech-Anderson 1987; Bech-Anderson *et al.* 1993; Evans *et al.* 1994; Green *et al.* 1995a; Green and Highley 1997). Oxalic acid



**Fig. 1.** Cup-plate assay of liquid culture filtrates of brown- and white-rot fungi. 1, *Coniophora puteana*; 2, *Postia placenta* Mad-698; 3, *Neolentinus lepideus*; 4, *Gloeophyllum trabeum*; 5, *P. placenta* ME-20; 6, *Serpula incrassata*; 7, *Antrodia xantha*; 8, *Laetiporus sulphureus* SH-27; 9, *L. sulphureus* Boat 206; 10, *A. carbonica*; 11, *A. serialis*; 12, *A. vaillantii*; 13, positive control; 14, *Phaseolus schweinitzii*; 15, *Fomitopsis pinicola*; 16, *F. palustris*; 17, *Trametes versicolor*; 18, *Phlebia brevispora*; 19, *Irpex lacteus*; 20, *Bjerkandera adusta*; 21, *Phanerochaete chrysosporium* ME-461; 22, *P. chrysosporium* ME-446; 23, *Ganoderma applanatum*; 24, *Xylobolus frustulatus*; 25, *Trichoderma harzianum*; 26, *Aspergillus niger*. The positive control consisted of a commercial preparation of *A. niger* pectinase.

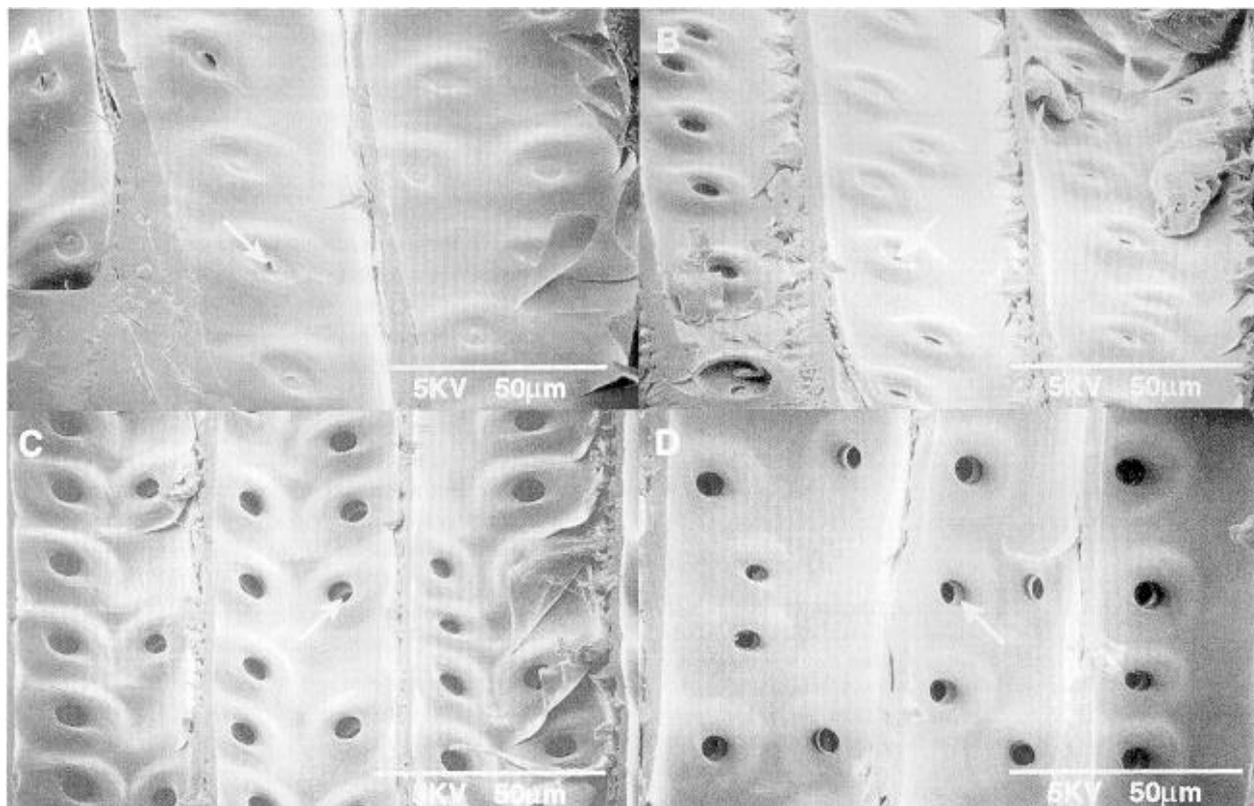
alone has been reported to solubilize pectin (Harlow 1930; Bishop 1955; Magro *et al.* 1984).

The *in vitro* culture conditions in this study were unique because the wood cores were incubated in liquid culture to permit enzyme analysis. According to most reports, brown-rot fungi, with the exception of Coniophoroid fungi, cannot depolymerize cellulose or cause wood weight loss in liquid culture (Highley 1974, 1975, 1977). One Coniophoroid brown-rot fungus, *Serpula incrassata*, has been shown to hydrolyze pit membranes and enlarge pit apertures of pine sapwood in soil-block tests (Green *et al.* 1995a, 1996). White-rot fungi have the enzymatic capacity to completely degrade cellulose of the pit margo in liquid culture due to a complete array of cellulases, which is consistent with Figure 2 (C and D) and which illustrates complete removal of the pit membrane. This is in concert with the gas permeability data in Table 1. In this study, the seven brown-rot fungi tested increased gas permeability of southern pine cores by approximately fourfold. When examined by SEM, the pit membranes in Figure 2 (A and B) appear to be damaged by exposure to brown-rot fungi in liquid culture causing rupture of some but not all membranes compared with intact membranes seen in negative control cores (Fig. 3A). Although some rupturing of pit membranes may occur during splitting and fracturing of wood, cores from each culture examined by SEM indicate that pit membranes were weakened or ruptured by exposure to the brown-rot fungi.

Similar results were observed for pinoid pits between ray parenchyma and tracheids (Jacobs-Young *et al.* 1998). Tschernitz (1973) showed similar pit morphology using Douglas-fir (*Pseudotsuga menziesii* (Mirb.) Franco) cores treated with commercial pectinase. Some weakened or eroded pit membranes in cores exposed to brown-rot fungi may have split during the drying process. It was previously shown in soil-block tests that brown-rot fungi can maximize the permeability of Douglas-fir and southern pine cores in 14 d by complete hydrolysis of the pit membrane (Green *et al.* 1995b; Green and Highley 1997).

Two brown-rot fungi, *N. lepideus* and *A. serialis*, produced no detectable oxalic acid under the culture conditions employed in this study. Similarly, the white-rot fungi *P. brevipespora* and *X. frustulatus* were uniformly negative for all tests under our culture conditions. Alternative hypotheses to explain how fungi that were negative for both oxalic acid and polygalacturonase colonize and degrade wood *in situ* during decay are (i) cellulase production alone, (ii) bore hole formation, or (iii) sodium polypectate may not induce endoPG and oxalic acid to the same degree that pectin does (Green *et al.* 1994).

This study demonstrates that white-rot fungi are able to hydrolyze the entire pit membrane in liquid culture and maximize permeability. However, white-rot fungi are equipped with exo-cellulases and peroxidases that are not present in most brown-rot fungi. Highley (1977) showed



**Fig. 2.** Scanning electron micrographs (5 kV) of Southern Pine bordered pits after exposure to brown-rot (A and B) and white-rot (C and D) decay fungi in liquid culture for 3 weeks. *Gloeophyllum trubeum* (A) and *Fomitopsis palustris* (B) show most bordered pit membranes are visible and ruptured (arrows). *Phanerochaete chrysosporium* (C) and *Irpex lacteus* (D) show complete solubilization of pit membranes; both apertures of pit pairs are visible (arrows).

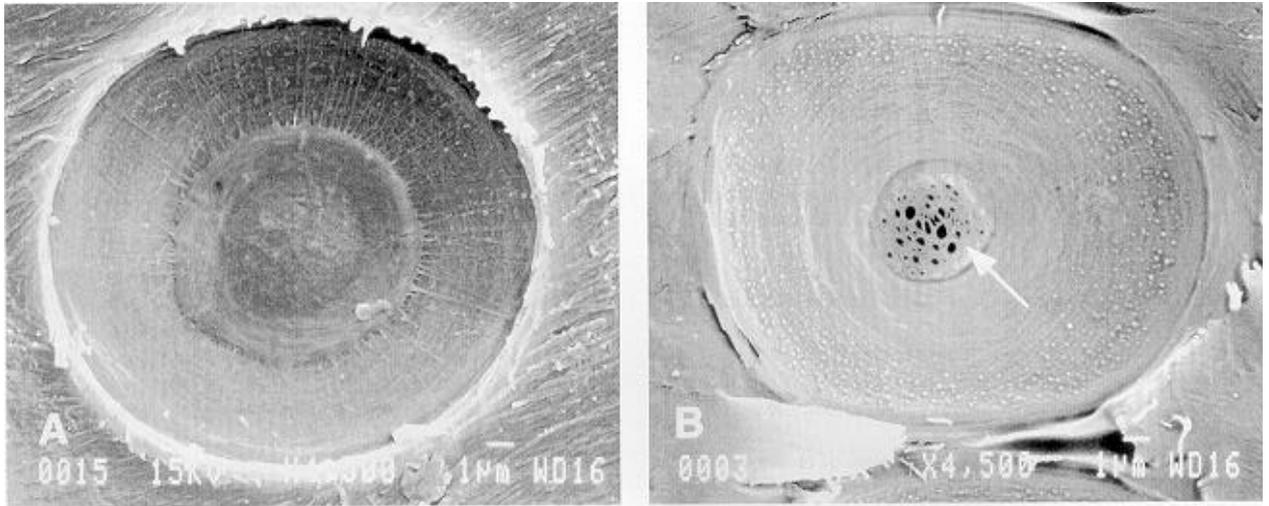


Fig. 3. Scanning electron micrographs (15 kV) of bordered pit membranes of pine: the aspirated torus of an intact bordered pit from a control pine core (A) and erosion of the pit torus by *Gloeophyllum trabeum* after 3 weeks in liquid culture (B). Arrow shows remaining cellulose macrofibrils of the pit membrane beneath the torus.

that enzyme preparations from the brown-rot fungus *Postia placenta* could not reproduce the effects of brown-rot fungi on wood or cotton cellulose as evidence that most brown-rot fungi employ a mechanism of cellulose degradation different from most other types of fungi. Coniophoroid brown-rot fungi are reported to solubilize dyed microcrystalline cellulose similar to white-rotters (Highley 1988). In this study, *C. puteana* did not demonstrate increased permeability compared with other brown-rot fungi. Tschernitz and Sachs (1973) showed that pectinase treatment of Douglas-fir hydrolyzes the pit torus leaving the margo intact, while cellulase treatment hydrolyzes the cellulose fibers of the margo leaving the torus unanchored. Permeability was shown to increase in both cases. Miltz (1993) demonstrated that commercial preparations of cellulolytic enzymes with some hemicellulolytic activities were more effective in pit degradation of spruce wood (*Picea* spp.) than pectinolytic enzyme preparations. The lack of oxalic acid accumulation in white-rot fungi (Table 1) is accounted for by their production of oxalate decarboxylase (ODC), which degrades oxalic acid to formate and carbon dioxide (Shimazono 1955). Although ODC has recently been discovered in a single brown-rot fungus (*Poria placenta* ME-20) as well (Micales 1995), it is apparently cell bound and difficult to measure.

Pectin degradation appears to be a key step in fungal colonization during incipient decay. Thus, inhibition of pectin degradation could be a target for new wood preservatives. Our results may help to explain how selective calcium precipitating agents, e.g., N,N-naphthaloylhydroxylamine (NHA), are able to protect wood from decay by both brown- and white-rot fungi (Green *et al.* 1997b). Scanning electron microscopy showed that NHA binds to the torus of the pit membrane and presumably prevents calcium chelation by oxalic acid, thereby preventing pit hydrolysis and colonization (Green *et al.* 1997a,b).

In summary, the results of this study show that most brown- and white-rot fungi (20 of 23 tested) have the ability

to remove pectin from pit membranes. The destruction of pit membranes during the decay process is probably accomplished by the hydrolysis of both pectin and cellulose.

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