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Induction of polygalacturonase and the formation of oxalic acid by pectin in brown-rot fungi†

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Extracellular polygalacturonase (PG) production was estimated in vitro, using liquid cultures of three species of brown-rot decay fungi *(Postia placenta, Gloeophyllum trabeum* and *Serpula incrassata),* by cup-plate assay, assay of reducing sugars, and decrease in viscosity. Although all three experimental assays demonstrated that PG was induced by pectin in all three fungi, decrease in viscosity gave the best correlation with decay capacity in soil block tests. PG activity, determined as an increase in reducing sugar activity, was greatest in *G. trabeum* and weakest in *S. incrassata.* The optimum pH for PG activity was between pH 2.5 and 4.5. Oxalic acid production was also enhanced by pectin and functioned synergistically with PG activity. We conclude that these fungi produce PG that is best induced by pectin and that PG activity exceeds production of xylanase and endoglucanase activity *in vitro.* Polygalacturonase is likely to act synergistically with oxalic acid to solubilize and hydrolyse the pectin in pit membranes and middle lamellae. Thus, production of PG and oxalic acid should facilitate early spread of hyphae and enhance the lateral flow of wood-decay enzymes and agents into adjacent tracheids and the wood cell wall, thus initiating the diffuse decay caused by brown-rot fungi.

Key words: Bordered pits, brown-rot fungi, polygalacturonase, oxalic acid, wood decay.

In a preliminary report on the formation of oxalic acid by brown-rot fungi, it was observed that pectin was a more efficient inducer of oxalic acid in *Postia placenta* than other hemicellulosic or cellulosic substrates (Green *et al.* 1994). Many studies on oxalic acid production by wood-decay fungi have focused on the possible role of oxalate in cellulose or hemicellulose hydrolysis (Schmidt *el al.* 1981; (Bech-Anderson 1987; Espejo & Agosin 1991; Shimada *et al.* 1994).

Studies of pectin-hydrolyzing enzymes in wood-decay fungi are scarce, probably because of the relatively low content (trace to 4%) of pectin in solid wood (Kertesz 1963). The low pectin content may be misleading in terms of the importance of pectin during early decay because most pectin is located in the central region or torus of the pit membranes and in the compound middle lamellae (CML) of the wood cell wall (Tschernitz & Sachs 1973; Murmanis & Chudnoff 1979; Militz 1993b).

The effects of treating conifers with commercial pectinase enzymes to improve penetration of preservatives have been thoroughly studied. Tschernitz (1973) demonstrated that the penetration of Douglas-fir sapwood increased following commercial pectinase (Pectinol) treatment. Gjovik (1977) showed increased penetration of creosote following *Trichoderma* colonization of conifer wood. The mould fungus *Trichoderma viridae* reduced the pectin content of fir wood to the same degree as pectinase enzymes (Sharma & Kumar 1979). Experiments have also been conducted to determine if removal of pit membranes by treatment with pectin-degrading enzymes would increase the flow rate of preservatives into the sapwood of pine tracheids (Nicholus & Thomas 1968; Militz 1993a).

The objective of the present study was to survey polygalacturonase (PG) activity in three taxonomically diverse brown-rot Basidiomycetes grown in liquid culture. In addition, degradation of the bordered pits in wood blocks decayed by *P. placenta* was studied by transmission electron microscopy.

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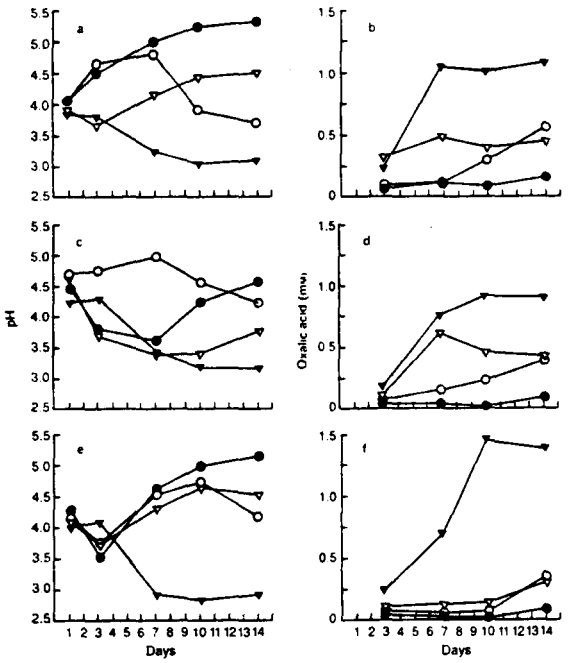

Figure 1. Changes in pH and oxalate concentrations in liquid cultures of brown-rot fungi supplemented with pectin (a, b), cellobiose (c, d) or both (e, f). ○—*P. placenta* MAD-696; ●—*P. placenta* ME-20; ▽—*G. trabeum* MAD-617; ▼—*S. incrassata* MAD-563.

Materials and Methods

Fungi

Four strains of brown-rot fungi [*Postia placenta* (Fr.) M. Las. et Lomb. MAD-698 and ME-20, *Gloeophyllum trabeum* (Pers. Fr.) MAD-617 and *Serpula incrassata* (Berk. & Curt.) Murr. MAD-563] were maintained on malt/agar. Pectin/agar plates were prepared by adding 1 g ammonium oxalate, 2 g citrus pectin (Sigma) and 3 g agar to 200 ml water (Highley 1973).

Liquid Cultures

Baileys minimal media (BMM; pH 4.0 to 4.4) (Highley 1973), 50 ml, was supplemented with 50 mg pectin, 50 mg cellobiose or 25 mg of each. Flasks were inoculated with ml 10-day-old liquid culture (BMM inoculated with malt/agar plug) and incubated at room temperature on a rotary shaker at 200 rev/min for 14 days. Aliquots (1 ml) were removed after 3, 7, 10, and 14 days for determination of pH, oxalate and PG. The fungi were also assessed for their ability to use pectin as a sole carbon source *on* 2% purified agar.

Oxalate, protein and Enzyme Assays.

Oxalic acid was estimated using a micro-adaptation of a commercial assay (Sigma).

Protein concentrations were measured using the commercial BCA protein assay reagent (Pierce Chemical, Cockeysville, MD), with BSA as standard.

Polygalacturonase was measured by a cup-plate assay (Dingle *et al.* 1953), a micro-adaptation of the Nelson-Somoygi reducing sugar assay, using 1% sodium polypectate as substrate (Green *et al.* 1989a), and by the reduction in viscosity of sodium polypectate and citrate pectin (Highley 1968, 1973), measured in a Cannon-Fenske viscometer.

Xylanase and cellulase were also estimated by reducing sugar assays, using 1% CM-cellulose (CMCase) and 1% xylan, respectively. (Green *et al.* 1989b)

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 40°C.

Soil Block Tests and Microscopy

Weight loss of southern yellow pine blocks (6 x 6 x 4 mm) was determined according to American Society for Testing and Materials D-2017 (Anon. 1991). Some blocks from the tests were also fixed in 4% glutaraldehyde in 0.1 M phosphate buffer, post-fixed in 1% OsO_4 in 0.1 M phosphate buffer, rinsed in water, dehydrated in ethanol and embedded in Quetol 651 (Electron Microscopy Sciences, PA). Thin sections were photographed on a Hitachi H300 transmission electron microscope.

Results

Experiments were performed to determine if a representative group of brown-rot Basidiomycetes could utilize pectin sole carbon source and if the pectin-degrading enzyme PG or oxalic acid could be detected in liquid cultures containing pectin, cellobiose or both.

Growth on Pectin/agar

All brown-rot fungi tested grew readily on purified agar with pectin as the sole carbon source at pH 4.22. *Gloeophyllum trabeum* (MAD-617) also formed rudimentary basidioma after 4 weeks.

Growth in Liquid Culture

All three brown-rot fungi grew readily in minimal salts medium with pectin, cellobiose or both carbon sources added (Figure 1). In general, pectin induced low pH and production of oxalic acid to a greater extent than did cellobiose, especially with S. *incrassata* (Figure 1). *Serpula incrassata* produced the most oxalate (1.44 mM) and the most acidic conditions (pH 2.77) in minimal salts medium supplemented with both pectin and cellobiose. Conversely, *P. placenta* ME-20 produced very low concentrations of oxalic acid, and the pH of the medium in which it was cultured increased from about 4.2 to between 4.5 and 5.0 after 2 weeks in culture.

Estimation of Polysaccharidases Activity

Nearly all enzyme activities peaked 10 days post-inoculation and generally followed the order PC > xylanase > CMCase (Figure 2). Polygalacturonase activity was usually greatest when the minimal salts medium was supplemented with pectin. The exception was ME-20, which produced more PG when both pectin and cellobiose were used. The greatest PG activity estimated was in *G. trabeum* grown on pectin alone.

To estimate the optimum pH range of the PG, filtrates of 10-day-old cultures were also tested for increased reduc-

Table 1. Estimation of polygalacturonase 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 loss in southern yellow pine wood blocks.

Fungus	Viscosity reduction ($10,000/t_{50}$)		Oxalate	Weight loss
	Sodium polypectate	Pectin	(μmol/mg dry wt)	(%)
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	50
S. incrassata MAD-563	7.4	2.3	48	50

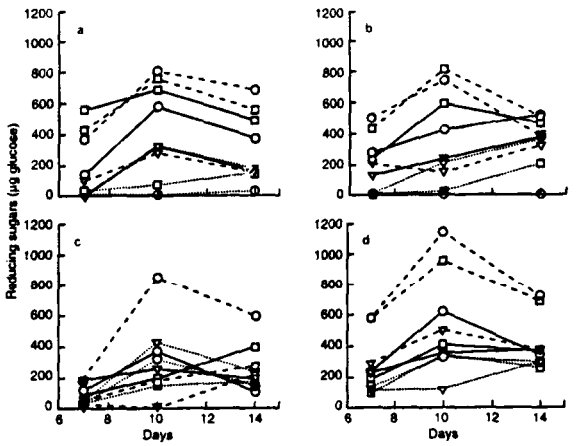

Figure 2. Induction of xylanase, CMCase of polygalacturonase in liquid cultures of the brown-rot fungi *P. placenta* MAD-698 (a), *P. placenta* ME-20 (b), *S. incrassata* MAD-563 (c) and *G. trabeum* MAD-617 (d) supplemented with pectin (○), cellobiose (▽) or both (□), as measured by increase in reducing sugars. — = xylanase, —— = cellulase, - - - = pectinase.

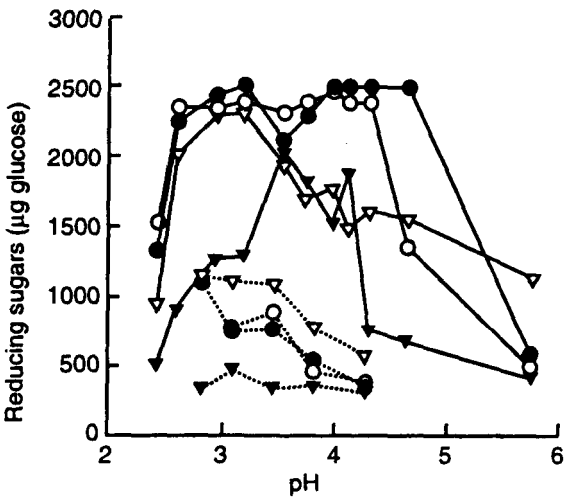

Figure 3. Optimal pH of polygalacturonase from *P. placenta* MAD-698 (○), *P. placenta* ME-20 (●), *G. trabeum* MAD-617 (▽) or *S. incrassata* MAD-563 (▼), estimated in oxalate (——) or citrate buffer (. . . .).

ing group activity, using 1% sodium polypectate as substrate in oxalate and citrate buffers ranging from pH 2.5 to 5.75 (Figure 3). *Postia placenta* and *G. trabeum* showed maximum production of reducing sugars and optimal activity from pH 2.8 to 4.5 in oxalate buffer. *Serpula incrassata* had relatively low activity and narrower optimal pH range (approx. 3.5 to 4.0). Citrate buffer always gave lower activities than oxalate (Figure 2).

When PG activity was estimated by the reduction in viscosity of pectin or polygalacturonate, *P. placenta* MAD-698 had the greatest activity (Table 1). The senescent isolate of *P. placenta,* ME-20, had low PG activity, low production of oxalate (μmol/mg) and gave very low weight loss of wood blocks in the soil block tests (Table 1). Although *S. incrassata MAD-563* had the least PG activity, it also had the greatest oxalate production and a decay capability similar to those of *G. trabeum* MAD-6 17 and *P. placenta* MAD-698.

When polygalacturonase activity was assessed by the cup-plate assay, it was visible in *P. placenta MAD-698* and ME-20 in cultures supplemented with pectin or pectin/cellobiose, but no precipitate was visible with cellobiose alone (Figure 4). Although *G. trabeum* showed maximum activity in cultures with pectin alone, *S. incrassata* only showed slight PG activity in such cultures.

Ultrastructural Examination of Wood Blocks

To confirm the attack of pectin-rich areas of wood during decay by brown-rot fungi, southern yellow pine wood blocks exposed to *P. placenta* MAD-698 and ME-20 were examined by transmission electron microscopy. Figure 5a shows *P. placenta* MAD-698 penetrating a tracheid-bordered pit membrane 10 days after colonization. Conversely, Figure 5b shows *P. placenta* ME-20 on both sides of an apparently intact pit membrane on day 31. The majority of pit membranes in blocks infested with strain ME-20 remained intact.

Discussion

The results from this study confirm that selected brown-rot fungi can use pectin as sole carbon source and that pectin is

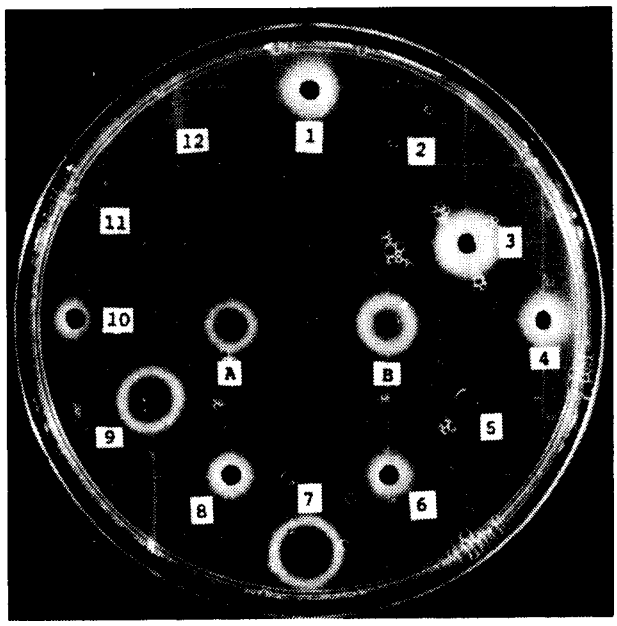

Figure 4. Cup-plate assay for polygalacturonase in liquid cultures of brown-rot fungi supplemented with pectin (P), cellobiose (C), or both (CP). 1 to *3-P.* placenta MAD-698 on P,C and CP, respectively; 4 to*6-P.* placenta ME-20 on P,C and CP, respectively; 7 to 9--G. *trabeum* MAD-617 on P,C and CP, respectively: 10 to 12—S. incrassata MAD-563 on P,C and CP, respectively; A-commercial pectinase (1 mg/ml); B-commercial cellulase (1 mg/ml).

Figure 5. Transmission electron micrographs of bordered pits of southern yellow pine in soil block test (a) 10 days post-inoculation with (a) *P. placenta* MAD-696, showing hyphae penetrating the bordered pit membrane (arrow), and (b) 31 days post-inoculation with *P. placerrta* ME-20, showing hyphae on both sides of the pit membrane but no penetration. Bar = 1 µm.

an efficient inducer of both oxalic acid and PG during growth of brown-rot fungi *in vitro.* The production of many extracellular pectinases by microorganisms is known to be induced by the presence of pectic materials in the culture medium (Galiotou-Panayotou *et al.* 1993). Micales (1994) showed that oxalic acid was induced in *P. placenta* MAD-698 when galacturonic acid was used as carbon source. The genera of brown-rot fungi used in the present study were selected to represent the three families of brown-rotters: Polyporaceae; Coniophoraceae; and Gloeophylaceae. The present results indicate that PG and oxalic acid function in concert to degrade pectic substances, especially those in the torus of bordered pit membranes (Tschernitz & Sachs 1973). They provide the first experimental evidence to support the suggestion that fungal decomposition of pectin is of importance during incipient wood decay by brown-rot fungi (Highley 1973; Espejo & Agosin 1991; Dutton *et al.* 1993; Evans *et al.* 1994). Three previous studies addressed the presence of pectinase enzymes in brown-rot fungi (King 1966; Highley 1976; Jennings 1991).

Their capacity to solubilize and hydrolyse pectic substances might provide brown-rot fungi with a competitive advantage over other fungi. Solubilization of the bordered pit membranes of tracheids during incipient brown-rot decay would not only provide non-lignified carbon sources (pectin, hemicellulose and cellulose) for growth but would also provide access to adjacent tracheids (Figure 6). Cowling (1961) and Wilcox (1968) reported that, during early brown-rot decay by *Postia* sp., the hyphae ramified through the entire wood block prior to 5% weight loss, largely by penetration of simple and bordered pits. In addition to the pit membranes, the pectin in the compound middle lamellae (Figure 6) may also be solubilized, thus weakening the structural cement between tracheids. Meier & Wilkie (1959) reported cellulose (36%), pectin (20%), and glucomannan (8%) in the CML of pine tracheids. Large gaps or pockets were seen during ultrastructural studies of brown-rot decay (Highley & Murmanis 1985).

The synergistic effects of oxalic acid and pectinase enzymes have been well established for numerous piant-pathogenic fungi, including *Sclerotium rolfsii* in beans (Bateman & Beer 1965), *Rhizoctonia* in potatoes (Arnadioha 1993) and *Aspergillus niger* in onion bulbs (Tanaka & Nonaka 1981). The oxalic acid serves to lower the pH of the host cells to

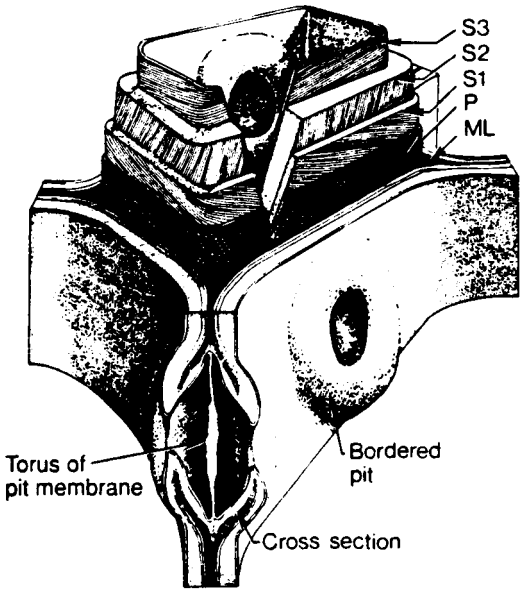

Figure 6. Bordered pit structure and its relationship to the layers of the wood cell wall of tracheids. Note that the bordered pit membrane is not aspirated. Cell wall layers: Middle lamella (ML), primary wall (P), S1, S2, S3.

a more favorable range for enzyme action and combines with the calcium associated with the pectin to facilitate hydrolysis by PC (Bateman & Beer 1965). Such effects have been observed during the hydrolysis of the middle lamella (Bateman & Beer 1965; Collmer & Keen 1986). A similar chain of events would be expected to occur during incipient brown-rot decay. The pH of wood blocks decreased rapidly to pH 1.7 during the first week of colonization with *P. placenta MAD-698,* concurrent with an increase in oxalic acid (Green *et al.* 1991, 1992). The optimum activity for the endopolygalacturonase of *Rhizopus* sp. LKN was 4.5 to 4.75, although some activity persisted above pH 7 (Elegado & Fujio 1994).

Transmission electron micrographs of wood blocks decayed by MAD-698 and ME-20 demonstrate that MAD-698 could penetrate the bordered pit membranes in 10 days, but ME-20 was not able to penetrate pits in 31 days. Although ME-20 demonstrated PG activity equivalent to MAD-698, when this was determined by reducing sugar assay, its ability to reduce the viscosity of sodium polypectate was much less than that of MAD-698. The MAD-698 enzyme sample did contain measurable oxalate, and ME-20 was previously shown to be a non-accumulator of oxalic acid upon colonizing wood blocks, there being no rapid decline in wood pH (Green *et al.* 1992). This inability to accumulate oxalic acid by ME-20 was recently shown by Micales (1995) to be the result of overproduction of extracellular oxalate decarboxylase. Thus, lack of oxalic acid accumulation may preclude effective hydrolysis of pectin by the PC of ME-20 and thus prevent penetration of wood blocks.

In summary, all the brown-rot fungi tested produced PC activity and oxalic acid in response to pectin as a carbon source. The greatest PG activity was demonstrated in *P. placenta* MAD-698 and *G. trabeum* MAD-617. The greatest oxalate production was in *S. incrassata* MAD-563. The weak decay isolate, ME-20, accumulated low oxalate and produced weak PG activity as shown by viscosity reduction. Oxalic acid was shown to facilitate PG activity, presumably by acting on the substrate as an effective chelator of Ca^{2+}, which results in solubilization of protopectin and ready access of pectic acid to hydrolyzing enzymes. Thus, pectin hydrolysis forms a positive feedback loop with oxalic acid production during incipient brown-rot decay, facilitating hydrolysis of bordered pit membranes and colonization of the entire wood block prior to measurable weight loss, as observed by Wilcox (1968). A reduction in either oxalic acid or pectin-hydrolyzing enzymes may interfere with initiation of the decay process, as observed with the senescent *P. placenta ME-20.*

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References

Amadioha, A.C. 1993 A synergism between oxalic acid and polygalacturonases in the depolymerization of potato tuber tissue. *World Journal of Microbiology and Biotechnology* **9**, 599–600.

Anon. 1991 Standard test method for assessing natural decay resistance. In *Annual Book of Standards*, D-2017-81. Philadelphia: American Society for Testing and Materials.

Bateman, D.F. & Beer, S.V. 1965 Simultaneous production and synergistic action of oxalic acid and polygalacturonase during pathogenesis by *Sclerotium rolfsii*. *Phytopathology* **55**, 204–211.

Bech-Anderson, J. 1987 *Production, Function and Neutralization of Oxalic Acid produced by the Dry-rot Fungus and other Brown-rot Fungi*. IRG/WP/1330. Stockholm, Sweden: The International Research Group on Wood Preservation.

Collmer, A. & Keen, N.T. 1986 The role of pectin enzymes in plant pathogenesis. *Annual Review of Phytopathology* **24**, 383–409.

Cowling, E.B. 1961 *Comparative Biochemistry of the Decay of Sweetgum Sapwood by White-rot and Brown-rot fungi*. WO-TB-1258. Washington DC.

Dingle, J., Reid, W.W. & Solomons, G.L. 1953 The enzymatic degradation of pectin and other polysaccharides. II. Application of the 'cup-plate' assay to the estimation of enzymes. *Journal of the Science of Food and Agriculture* **4**, 149–155.

Dutton, M.V., Evans, C.S., Atkey, P.T. & Wood, D.A. 1993 Oxalate production of Basidiomycetes, including the white-rot species *Coriolus versicolor* and *Phanerochaete chrysosporium*. *Applied Microbiology and Biotechnology* **39**, 5–10.

Elegado, F.B. & Fujio, Y. 1994 Purification and some properties of endopolygalacturonase from *Rhizopus* sp LKN. *World Journal of Microbiology and Biotechnology* **10**, 256–259.

Espejo, E. & Agosin, E. 1991 Production and degradation of

oxalic acid by brown-rot fungi. *Applied and Environmental Microbiology* **57**, 1980–1986.

Evans, C.S., Dutton, M.V., Guillen, F. & Veness, R.G. 1994 Enzymes and small molecular mass agents involved with lignocellulose degradation. *FEMS Microbiology Reviews* **13**, 235–240.

Galiotou-Panayotou, M., Rodis, P. & Kapantai, M. 1993 Enhanced polygalacturonase production by *Aspergillus niger* NRRL-364 grown on supplemented citrus pectin. *Letters in Applied Microbiology* **17**, 145–148.

Gjovik, L.R. 1977 Pretreatment molding of southern pine: its effects on the permanence and performance of preservatives exposed to seawater. *Proceedings of the American Wood Preservation Association* **73**, 142–153.

Green, F., Clausen, C.A. & Highley, T.L. 1989a Adaptation of the Nelson–Somoygi reducing sugar assay to a microassay using microtiter plates. *Analytical Biochemistry* **182**, 197–199.

Green, F., Clausen, C.A., Micales, J.A., Highley, T.L. & Wolter, K.E. 1989b Carbohydrate-degrading complex of the brown-rot fungus *Postia placenta*: purification of β-1,4-xylanase. *Holzforschung* **43**, 25–31.

Green, F., Larsen, M.J., Hackney, J.M., Clausen, C.A. & Highley, T.L. 1992 Acid-mediated depolymerization of cellulose during incipient brown-rot decay by *Postia placenta*, In *Biotechnology in Pulp and Paper Industry, 5th International Conference*, eds Kuwahar, M. & Shimada, M. pp. 267–272. Tokyo: Uni.

Green, F., Larsen, M.J. & Highley, T.L. 1994 *Hemicellulosic Induction of Oxalic acid in* Postia placenta. IRG/WP/94-10060. Stockholm. Sweden: The International Research Group on Wood Preservation.

Green, F., Larsen, M.J., Winandy, J.E. & Highley, T.L. 1991 Role of oxalic acid in incipient brown-rot decay. *Material und Organismen* **26**, 191–213.

Highley, T.L. 1968 Comparison of pectic enzymes in resistant and susceptible mints infected by *Verticillium dahliae*. PhD Dissertation, Oregon State University.

Highley, T.L. 1973 Influence of carbon source on cellulase activity of white-rot and brown-rot fungi. *Wood and Fiber Science* **5**, 50–58.

Highley, T.L. 1976 Hemicellulases of white- and brown-rot fungi in relation to host preferences. *Material und Organismen* **11**, 33–46.

Highley, T.L. & Murmanis, L. 1985 Micromorphology of degradation of western hemlock and sweetgum by the brown-rot fungus *Postia placenta*. *Holzforschung* **39**, 73–79.

Jennings, D.H. 1991 The physiology and biochemistry of the vegetative mycelium. In Serpula lacrymans—*Fundamental Biology and Control Strategies*, eds Jennings, D.H. & Bravery, A.F. pp. 55–81. New York: John Wiley & Sons.

Kertesz, Z.I. 1963 Pectic polyuronides. In *Comprehensible Biochemistry*, eds Florkin, M. & Stotz, E.H. pp. 233–245. Amsterdam: Elsevier.

King, N.J. 1966 The extracellular enzymes of *Coniophora cerebella*. *Biochemistry Journal* **100**, 784–792.

Meier, H. & Wilkie, K.C.B. 1959 The distribution of polysaccharides in the cell wall of tracheids of pine (*Pinus silvestris* L.). *Holzforschung* **13**, 177–182.

Micales, J.A. 1994 Induction of oxalic acid by carbohydrate and nitrogen sources in the brown-rot fungus *Postia placenta*. *Material und Organismen* **28**, 197–207.

Micales, J.A. 1995 Oxalate decarboxylase in the brown-rot wood decay fungus *Postia placenta*. *Material u. Organismen* **3**, (in press).

Militz, H. 1993a Changes in the microstructure of spruce wood (*Picea abies* L. Karst) through treatment with enzyme preparations, alkali and oxalate. *Holzforschung und Holzverwertung* **45**, 50–53.

Militz, H. 1993b The enzymatic decomposition of neutral and acid polysaccharides from spruce wood. *Wood Science and Technology* **28**, 9–22.

Murmanis, L. & Chudnoff, M. 1979 Lateral flow in beech and birch as revealed by electron microscopy. *Wood Science and Technology* **13**, 79–87.

Nicholus, D.D. & Thomas, R.J. 1968 The influence of enzymes on the structure and permeability of loblolly pine. *Proceedings of the American Wood Preservation Association* **64**, 70–76.

Schmidt, C.J., Whitten, B.K. & Nicholas, D.D. 1981 A proposed role for oxalic acid in non-enzymatic wood decay by brown-rot fungi. *Proceedings of the American Wood Preservation Association* **77**, 157–164.

Sharma, M. & Kumar, S. 1979 Degradation of wood pectin by microorganisms. *International Journal of Wood Preservation* **1**, 87–90.

Shimada, M., Ma, D.B., Akamatsu, Y. & Hattori, T. 1994 A proposed role of oxalic acid in wood decay systems of wood-rotting basidiomycetes. *FEMS Microbiology Reviews* **13**, 285–296.

Tanaka, K. & Nonaka, F. 1981 Synergistic action of oxalic acid and pectolytic enzyme on the rot of onion bulb caused by *Aspergillus niger*. *Annals of the Phytopathological Society of Japan* **47**, 166–174.

Tschernitz, J.L. 1973 Enzyme mixture improves creosote treatment of kiln-dried Rocky Mountain Douglas-fir. *Forest Products Journal* **23**, 30–38.

Tschernitz, J.L. & Sachs, I.B. 1973 Observations of microfibril organizations of Douglas-fir bordered pit-pair membranes by scanning electron microscopy. *Wood and Fiber* **6**, 332–340.

Wilcox, W.W. 1968 *Changes in Wood Microstructure through Progressive Stages of Decay*. FPL-RP-70. Madison, WI: Forest Products Laboratory, Forest Service, US Department of Agriculture.

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