

THE INTERNATIONAL RESEARCH GROUP ON WOOD PRESERVATION

Section 1

Biology

HYDROLYSIS OF BORDERED PITS DURING COLONIZATION
OF CONIFERS BY BROWN-ROT DECAY

BY

F. Green III, J. Tschernitz, T.A. Kuster and T.L. Highley

U.S. Department of Agriculture, Forest Service
Forest Products Laboratory
One Gifford Pinchot Dr.
Madison, WI 53705-2398 U.S.A.

Paper prepared for the 26th Annual Meeting
Helsingør, Denmark
11-16 June 1995

IRG Secretariat
Box 5607
S-114 86 Stockholm
Sweden

HYDROLYSIS OF BORDERED PITS DURING COLONIZATION
OF CONIFERS BY BROWN-ROT FUNGI

BY

Frederick Green III, John L. Tschernitz, Thomas A. Kuster and Terry L. Highley

U.S. Department of Agriculture, Forest Service
Forest Products Laboratory¹
One Gifford Pinchot Dr.
Madison, WI 53705-2398 U.S.A.

ABSTRACT

Brown-rot decay results in rapid reduction in degree of polymerization (DP) of holocellulose with concomitant strength loss (MOR) without removing lignin. Development of new methods of wood protection will require focusing on early events in the sequence of depolymerization. Bordered pit membranes (sapwood) represent a readily available source of non-lignified carbohydrate, ie. pectin and cellulose. Commercial pectinases (Pectinol) and Trichoderma sp. have been shown to degrade pit membranes and increase penetration of preservatives. Brown-rot fungi have previously been shown to produce oxalic acid (OA) during the decay process. Plant pathogens have been shown to degrade pectin by the synergistic action of OA and polygalacturonase (PG). The OA solubilizes the pectin by chelating the Ca⁺⁺ and the PG hydrolyses the α -1,4 linkages. We have demonstrated the ability of P. placenta, G. trabeum and S. incrassata to use pectin as a sole carbon source and to produce OA and PG on both liquid media and wood. A. niger and Trichoderma sp. also produce PG on wood but no OA or weight loss. The optimal pH of brown-rot polygalacturonase activity is circa 4.0. As the pH of the wood drops below pH 4, due to acid production during decay, there is a progressive decrease in PG activity and the possibility of acid catalyzed hydrolysis of pit membranes is suggested by increased permeability. We hypothesize that pectin utilization is an essential step during incipient brown-rot decay which helps to initiate fungal metabolism and promote the spread of fungal hyphae between tracheids.

¹The Forest Products Laboratory is maintained in cooperation with the University of Wisconsin. This article as written and prepared by U.S. Government employees on official time, and it is therefore in the public domain and not subject to copyright.

INTRODUCTION

Brown-rot decay can be characterized by diffusion of low molecular weight decay agents into the wood cell wall at large distances from the fungal hyphae in the cell lumen. In order for fungal hyphae to colonize the lumen of every cell, either penetration of bordered pit membranes or formation of bore holes must provide access to adjacent cells. Increasing the permeability of the wood by degrading the bordered pit membranes serves both to disseminate the hyphae and the decay agents.

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 (Shanley 1993). The low pectin content may be misleading in terms of importance during early decay for the following reasons. Anatomically, 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 (Militz 1993b; Murmanis & Chudnoff 1979; Tschernitz & Sachs 1973).

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 sapwood of Douglas-fir increased following commercial pectinase (Pectinol-L59RH) treatment. Lindgren (1952) and Gjovik (1977) showed increased penetration of preservatives following Trichoderma colonization of conifer wood. The mold 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 (Militz 1993a; Nicholas & Thomas 1968).

Recently, we reported the in vitro induction of polygalacturonase (PG) and formation of oxalic acid (OA) to pectin in brown-rot fungi (Green et al. , 1995). The present work was undertaken to examine relationships between brown-rot decay, oxalic acid and bordered pit degradation during brown-rot decay of southern pine.

MATERIALS AND METHODS

Fungi: Brown-rot fungi Postia placenta (Fr.) M.Lars. et Lomb., [MAD-698 and ME-20]; Gloeophyllum trabeum (Pers. Fr.) (MAD-617), Serpula incrassata (Berk. & Curt.) Murr. (MAD-563), and the mold fungi Aspergillus niger and Trichoderma sp. were maintained on malt agar. Pectin agar plates were prepared by adding

1 g ammonium oxalate plus 2 g citrus pectin (Sigma), plus 3 g agar to 200 cc water.

ASTM soil block test: Weight loss of southern pine blocks (18x18x4mm) was determined according to American Society for Testing and Materials D-2017 (ASTM 1991). Other similar blocks were split longitudinally and immersed in aqueous 0.1% Triton X-100 (2cc/2 blocks) at 7, 14, 21 & 28d intervals. All pH estimates were made on these wood extracts after 4h agitation.

Oxalate assay: Oxalic acid was estimated by a microadaptation of a commercial assay (Sigma, St. Louis, MO).

Protein assay: Protein concentrations were measured using the BCA protein assay reagent (Pierce Chemical Co., Cockeysville, MD) with bovine serum albumin as standard.

Enzyme assays: Polygalacturonase was measured by a microadaptation of the Nelson-Somoygi reducing sugar assay using 1% sodium polypectate as substrate (Green et al . 1989a).

Gas permeability: Douglas fir cores (64mm diam) were bored from green sapwood (Tschernitz 1973). Cores were either treated for 4h with 2%/vol Pectinol-L59(Rohm and Haas) alone or in combination with ammonium oxalate or hexametaphosphate (Calgon). Additional untreated cores were placed into soil block tests. Permeability was measured and calculated after the method of Milota et al ., (1995).

Electron microscopy: For scanning electron microscopy (SEM) decayed samples (14d) of Southern pine were extracted with 0.1% Triton X-100 for 4 hours, rinsed in distilled water for 24 hours, placed in 100% ethanol for 48 hours and critical point dried using a Balzers CPD 020. The samples were treated with Triton X-100 to remove the fungal slime layer on the pit torus and margo. Frozen Douglas fir samples were oven dried at 160°F. All samples were split in the radial plane, mounted on stubs and gold coated using a Denton sputter coater Desk-1. The samples were examined and photographed using a JEOL 840 scanning electron microscope.

RESULTS

The results from wood block experiments are presented in Figures 1, 2, and 3. The pH of wood extracts decreased only with P . placenta MAD-698, G . trabeum MAD-617 and S . incrassata MAD-563 (Fig. 1a). Oxalic acid accumulated in only P . placenta MAD-698 and S . incrassata MAD-563 (Fig 1b). Protein rose

significantly in P . placenta MAD-698, G . trabeum MAD-617 and S . incrassata MAD-563 (Fig 1C). However, this to some extent indicates wood degradation and release of interference compounds. All six fungi had measurable polygalacturonase, but in general, values for the non-decay fungi were high and the brown-rot decay fungi low (Fig. 1d).

The optimal pH of the polygalacturonases (PG)isolated from coil block tests are shown in Fig 2. All six fungi showed peak PG activity circa pH 4.0, with little or no activity at pH 2.5 or 5.5 when tested by reducing sugars.

Weight loss of southern pine wood blocks are shown in Fig. 3.

Gas permeability of 6.4mm Douglas fir sapwood cores following 4h treatment with 2%/vol Pectinol-L59 alone or with ammonium oxalate or Calgon are shown in Fig. 4.

Gas permeability of 6.4mm Douglas fir cores following incubation with brown-rot fungi are shown in Table 1:

Table 1. Effect of brown-rot decay on longitudinal gas permeability and weight loss of Douglas-fir sapwood cores (6.5mm)

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

¹Darcys (Milota 1994) ²pits aspirated

Scanning electron microscopy of bordered pits following 2 week incubation with brown-rot fungi are shown in Fig. 5. Normal aspirated pit is shown in Fig. 5-1. Margo of pit in Fig. 5-2 appears damaged by P . placenta MAD-698. Torus of pit in Fig. 5-4 appears degraded by G . trabeum MAD-617. Pit membranes are absent and pit apertures are enlarged by S . incrassata MAD-563 (Fig. 5-5) when compared with Fig. 5-4 (G . trabeum). All pits appear to remain intact after colonization by P . placenta ME-20 (Fig. 5-6).

DISCUSSION

This paper provides evidence for degradation of bordered pit membranes during incipient brown-rot decay. All brown-rot fungi tested produced polygalacturonase. Postia and Serpula also accumulate oxalic acid which is shown to work synergistically with Pectinol to increase wood permeability (Tschernitz 1973). The gas permeability of Douglas-fir sapwood cores increased to near

maximum (ca. 11 darcys) following colonization and attack by three brown-rot fungi. Ultrastructural examination of southern pine pit membranes by scanning electron microscopy following soil block tests with brown-rot fungi revealed damage to pit membranes and in one fungus (MAD-563) enlargement of pit apertures; this isolate of Serpula incrassata is a very aggressive brown-rot fungus causing rapid weight loss. Pit apertures may be degraded by the potent extracellular cellulose-depolymerizing enzymes produced by this fungus (Kleman-Leyer 1994).

Little attention has been focused on degradation of bordered pit membranes by brown-rot fungi (Green et al. ., 1995, and Daniels et al. ., 1994). This is surprising since Cowling (1961) and Wilcox (1968) reported that hyphae ramified throughout the entire wood block prior to 5% weight loss. Penetration of the numerous bordered pit pairs (Fig. 6) was considered the most likely avenue of attack for adjacent tracheids. In point of fact--concepts of pectin hydrolysis came the long way around upon rediscovery of the potential importance of oxalic acid during brown-rot decay (Green et al. ., 1991, Dutton et al. ., 1993, Evans et al. ., 1994). It has been previously shown, basically with plant pathogens, that oxalic acid and pectin degrading enzymes function synergistically (Bateman and Beer 1965). Oxalic acid is believed to act by direct disintegration of pectic substances (Bishop 1955) and by lowering the pH of host tissues and rendering the calcium-pectate complexes more susceptible to hydrolysis (Magro et al. ., 1984).

The role of pH in hydrolysis of bordered pit membranes gets more complicated when the optimal range of pH of polygalacturonases is considered (Fig 2). The optimal pH of polygalacturonase is circa 4.0 and as the pH drops, activity diminishes to nearly zero at pH=2.5. However, as the pH in Fig. 4 was made more acidic with HCl, wood permeability rose to about 5.0, even though HCl does not increase wood permeability alone (unpublished results). Although oxalic acid is an effective calcium binding compound, no similar role can be claimed for hydrochloric acid. Therefore, there may be an additional synergistic effect between an acidic pH and polygalacturonase activity not yet understood--or possibly a different enzyme?

The synergistic action of oxalic acid and cell wall degrading enzymes, especially endopolygalacturonase, is well documented in plant pathology (Maxwell and Lumsden 1970). However, as can be seen in Fig. 1b, all brown-rot fungi do not accumulate oxalic acid during decay. Specifically, G. trabeum produces detectable oxalic acid early, but later it is either degraded or converted to insoluble calcium salt (Espejo and Agosin 1991). Nevertheless, during decay with G. Trabeum, the pH goes down (Fig. 1a) and wood weight loss increases (Fig. 4).

P. placenta ME-20 does not lower PH, cause wood weight loss, or accumulate oxalic acid. The latter is due to overproduction of oxalate decarboxylase, thought until recently to only be produced by white-rot fungi (Micales 1995). Even with production of Polygalacturonase, permeability of wood does not increase and pits are not degraded most likely because of no oxalic acid (Fig. 5-6). This inability of ME-20 to degrade pits has also been observed by TEM (Green et al. ., 1995).

In spite of their reputation for producing commercial pectinases and cellulases respectively, A. niger and Trichoderma sp. did not lower wood pH, increase the

permeability of wood (unpublished results), or cause wood weight loss. This is surprising since A . niger is listed as a primary producer of oxalic acid in Merck Index (9th Edition). These results underscore the importance of the acidic conditions induced during brown-rot decay and that enzymes are not the key to wood degradation. Trichoderma sp. have been shown by previous authors to increase wood permeability by degrading pits (Sprading, 1936; Gjovik, 1977; Sharma and Kumar 1979). However, under our culture conditions we did not get this result?

Synergistic action of Polygalacturonase and oxalic acid is clearly not the whole story. In closely examining the attack of brown-rot fungi on bordered pits in Fig. 5 we observed that endoglucanases may also play a role in degradation of the margo which is comprised of cellulose microfilaments (Fig. 5-2). Thus multiple strategies may be required to insure penetration of pit membranes? Clearly, polygalacturonase activity decreases as pH becomes more acidic (Fig. 2). Xylanase is still fully active at the low pH's actually measured during brown-rot decay by P . placenta MAD-698 (Green et al ., 1989b, Green et al ., 1991).

In summary, experimental evidence is provided for rapid degradation of bordered pit membranes during incipient decay. This is likely accomplished by a combination of oxalic acid solubility, hydrolysis of the torus by polygalacturonase, and hydrolysis of the margo by endoglucanases. Hydrolysis of bordered pit membranes i) facilitates fungal growth with a source of non-lignified carbohydrate ii) induces polygalacturonase and formation of oxalic acid iii) increases the permeability of the wood to aqueous decay agents (Clausen et al ., 1995) and iv) provides for rapid penetration of fungal hyphae into adjacent cells. The inability to degrade and penetrate pit membranes appears to contribute to the inability of ME-20 to degrade wood.

REFERENCES

- Amadioha, A.C. 1993 A synergism between oxalic acid and polygalacturonases in the depolymerization of potatoe tuber tissue. World Journal of Microbiology and Biotechnology 9, 599-600.
- ASTM. 1991 Standard test method for assessing natural decay resistance. ASTM D-2017-81. Annual Book of Standards. American Society for Testing and Materials, Philadelphia, PA.
- 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. International Research Group on Wood Preservation. IRG/WP/1330.
- Bishop, C.T. 1995 Carbohydrates of sunflower heads, Canadian Journal of Chemistry 33:1521-1529.
- Clausen, C.A. and L. Ferge. 1995 Dimensional lumber model demonstrates the sensitivity of the particle capture immunoassay in early detection of brown-rot decay, International Research Group on Wood Preservation Doc. No. IRG/WP/
- Cowling, E.B. 1961 Comparative biochemistry of the decay of sweetgum sapwood by white-rot and brown-rot fungi. WO-TB-1258. Washington, DC: 79 pp.
- Daniel, G., Singh, A, and T. Nilsson. 1994. Ultrastructural and immunocytochemical studies on the window and bordered pit membranes of Pinus sylvestris L. Third Pacific Regional Wood Anatomy Conference, Rotorua, p.201.
- 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 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.
- Gjovik, L.R. 1977 Pretreatment molding of Southern Pine: its effects on the

- permanance and performance of preservatives exposed to seawater. American Wood Preservation Association Proceedings 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 B-1,4-xylanase. Holzforschung 43, 25-31.
- 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.
- 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. Kuwahar, M, Shimada, M., eds. Uni Publishers, LTD, Tokyo, Japan.
- Green, F., Larsen, M.J. & Highley, T.L. 1994 Hemicellulosic induction of oxalic acid in Postia placenta. International Research Group on Wood Preservation IRG/WP/94-10060.
- Green, F., Clausen, C.A., Kuster, T. and Highley, T.L. 1995. Induction of polygalacturonase and formation of oxalic acid by pectin dujring incipient brown-rot decay. World J. of Micro. Biotech, in press.
- Kleman-Leyer, K, and Kirk, T.K. 1994. Three native cellulose depolymerizing sudoglucanases from solid substrate cultures of the brown-rot fungus Meruliporia (Serpula) incrassata. Applied Envir. Micro. 60:2839-2845.
- Lindgren, R.M. 1952. Permeability of Southern Pine as affected by mold and other fungus infection, AWPA Proceedings 48:1-11.
- Magro, P., Marciano, P. and DiLenna, P. 1984. Oxalic acid production and its role in pathogenesis of Sclerotinia sclerotiorum. FEMS Microbiology Letters 24, 9-12.
- Maxwell, D.P. and Lumsden, R.D. 1970. Oxalic acid production by Sclerotinia sclerotiorum in infected bean and in culture. Phytopathology 60, 1395-1398.
- Micales, J.A. 1994a. Induction of oxalic acid by carbohydrate and nitrogen sources in the brown-rot fungus Postia placenta. Materials und Organismen 28, 197-207.
- Micales, J.A. 1995. Oxalate decarboxylase in the brown-rot fungi Postia placenta. Material und Organismen (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.

Milota, MR, Tschernitz, JL, Verill, SP and T. Mianowski. 1995 Gas permeability of Plantation Loblolly pine. Wood and Fiber Sci. 27: 34-40.

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. 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.

Shanley, N.A., Van Den Broek, L.A.M., Voragen, A.G.J. and Coughan, M.P. 1993. Isolation and characterization of an endopolygalacturonase from Phanerochaete chrysosporium. J. Biotech. 28:179-197.

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.

Sprading, Mae. 1936. Penetration of Trichoderma lignorum into sapwood of Pinus taeda. J. Agriculture Res. 52: 541-546.

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 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 on 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: U.S. Department of Agriculture, Forest Service, Forest Products Laboratory.

Figure Legends

Figure 1--Estimates of pH, oxalic acid, protein and PG on wood extracts of Southern Pine following inoculation with Postia placenta MAD-698, P . placenta ME-20, G . trabeum MAD-617, S . incrassata MAD-563, A . niger and T . viridae.

Figure 2--Optimal pH of polygalacturonase wood extracts from Southern Pine following fungal colonization in ASTM Soil Block Test by reducing sugar assay. 0.5% oxalate buffer (2.5-4.2) acetate buffer (4.2-6.0).

Figure 3--Per cent weight loss of southern pine following soil block test.

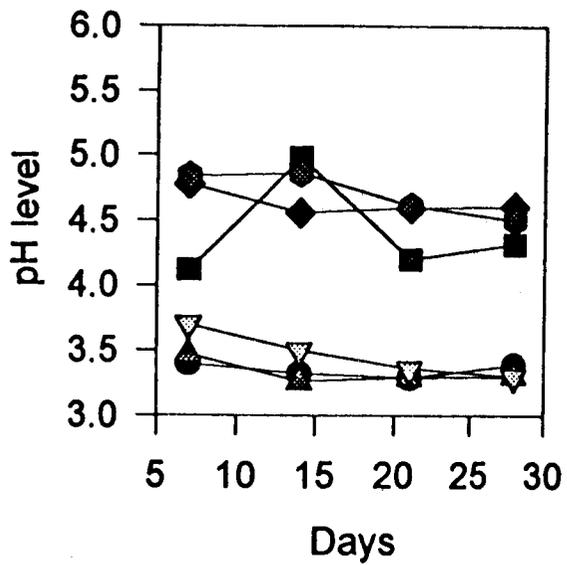
Figure 4--Gas permeability (darcys) OF. 6.5mm Douglas Fir cores following 4h treatment with 2%/vol Pectinol, or 2% Pectinol plus 2% oxalate (NH₄) or 2% hexametaphosphate.

Figure 5--Scanning electron micrographs of Southern Pine bordered pits following brown-rot decay (14d):

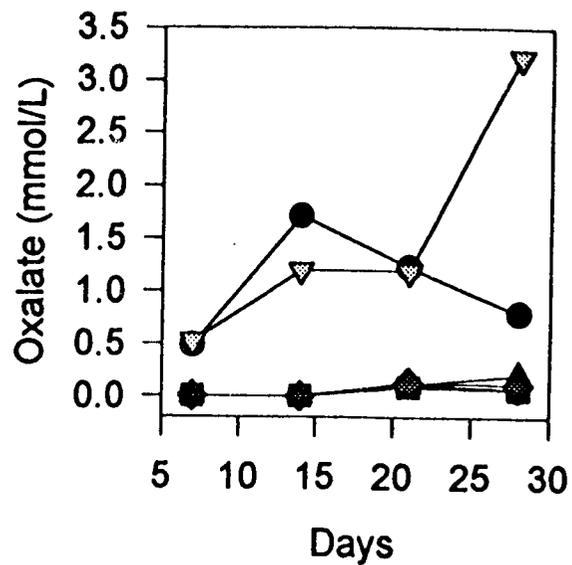
- 5-1 control
- 5-2 P . placenta MAD 698
- 5-3/5-4 G . trabeum MAD 617
- 5-5 S . incrassata MAD 563
- 5-6 P . placenta ME20

Figure 6--Bordered pit structure and its relationship to the layers of the wood cell wall of tracheids. Bordered pit membrane not aspirated.

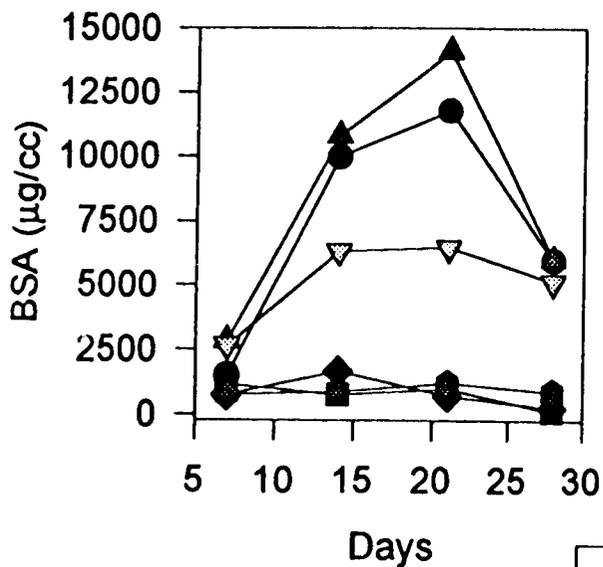
Changes in pH level



Oxalic Acid



BCA Protein



Reducing Sugars for Polygalacturonase

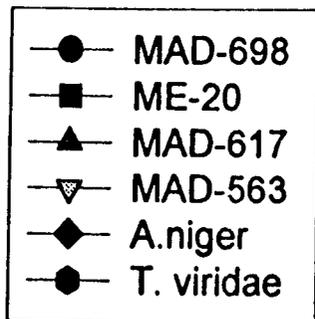
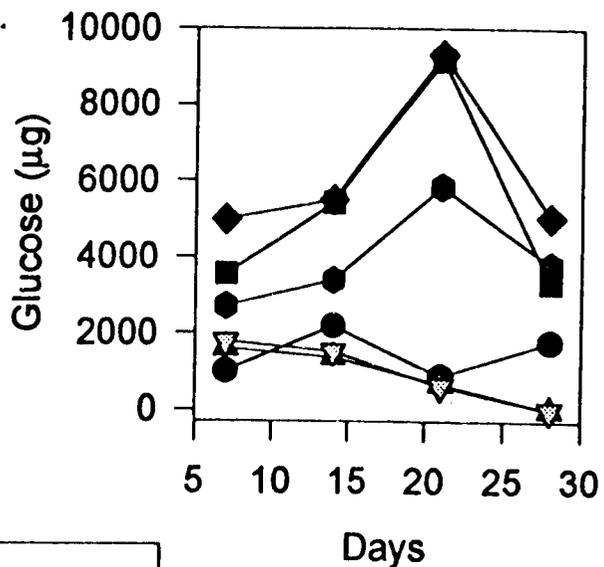


Figure 1.

**pH level vs. Reducing sugars for polygalacturonase
Day 5 / Southern Pine / 1-95**

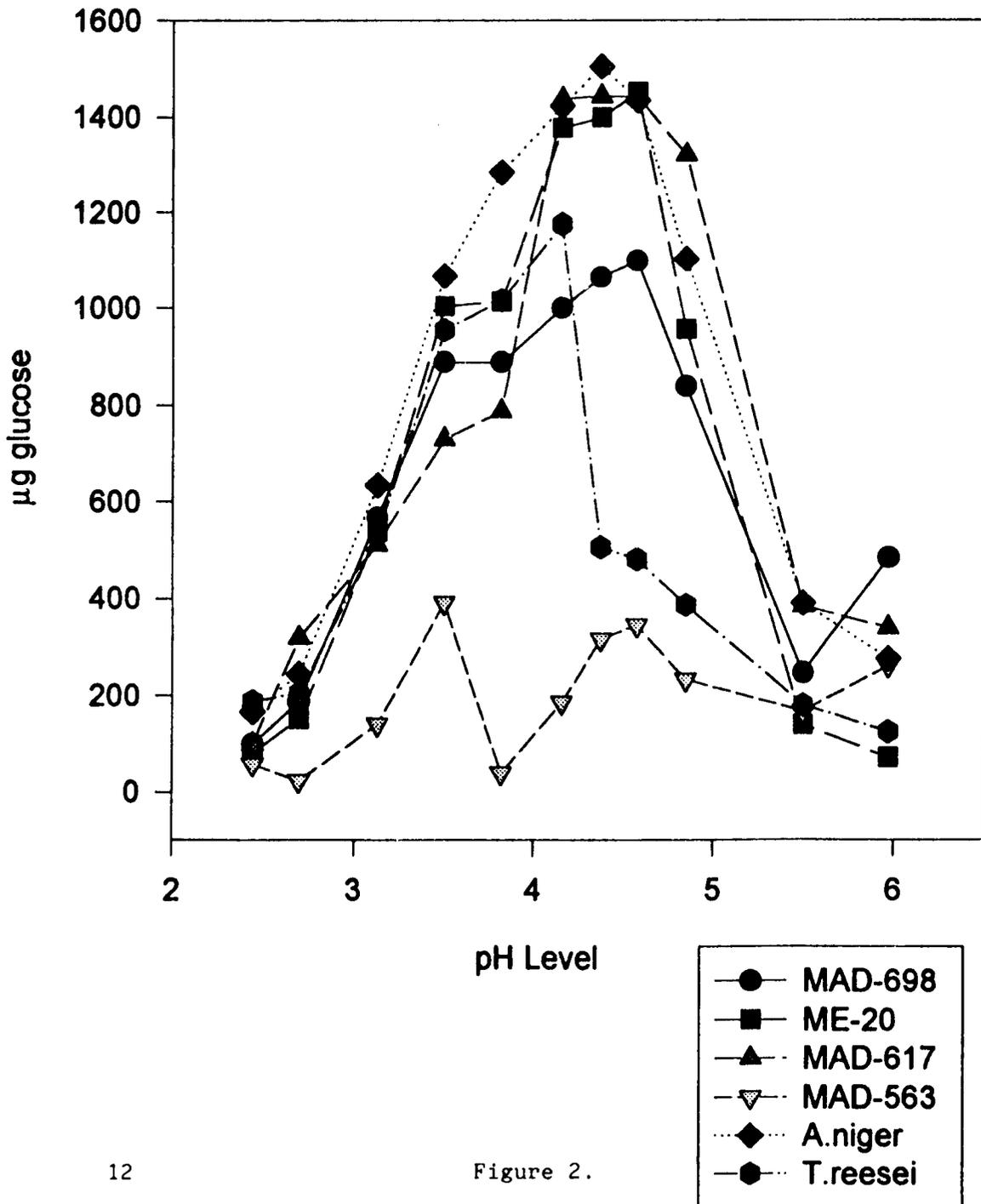


Figure 2.

Percent Weight Loss of Southern Pine

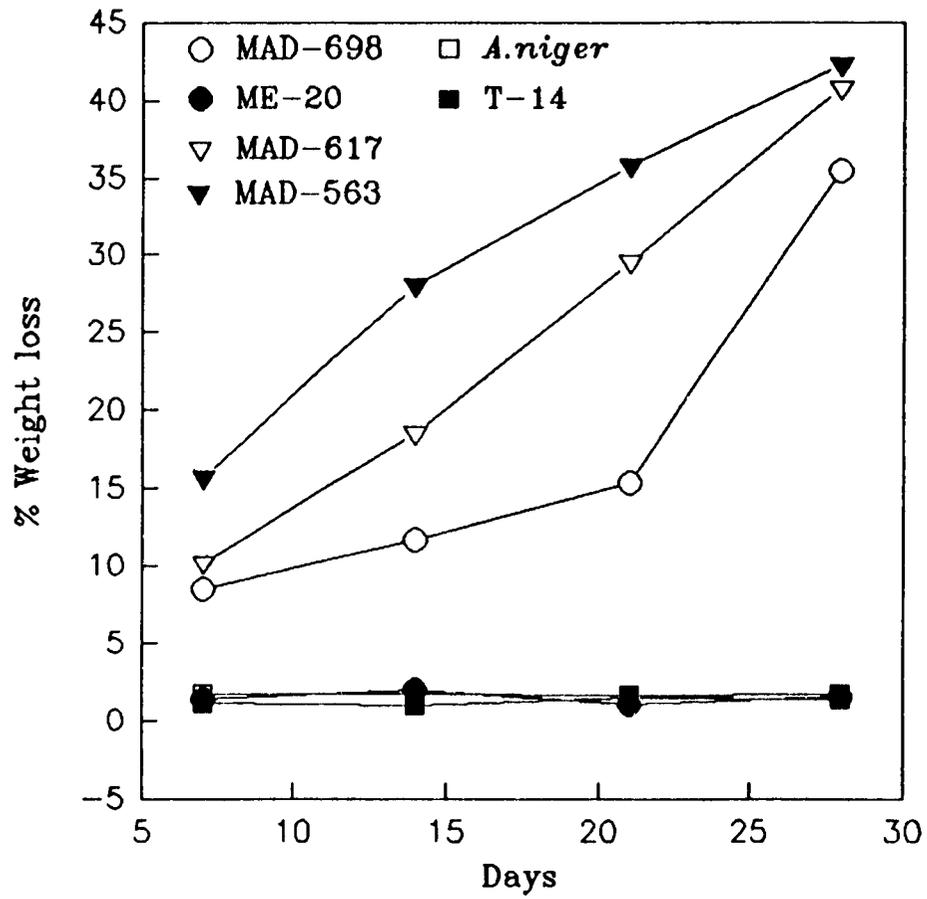
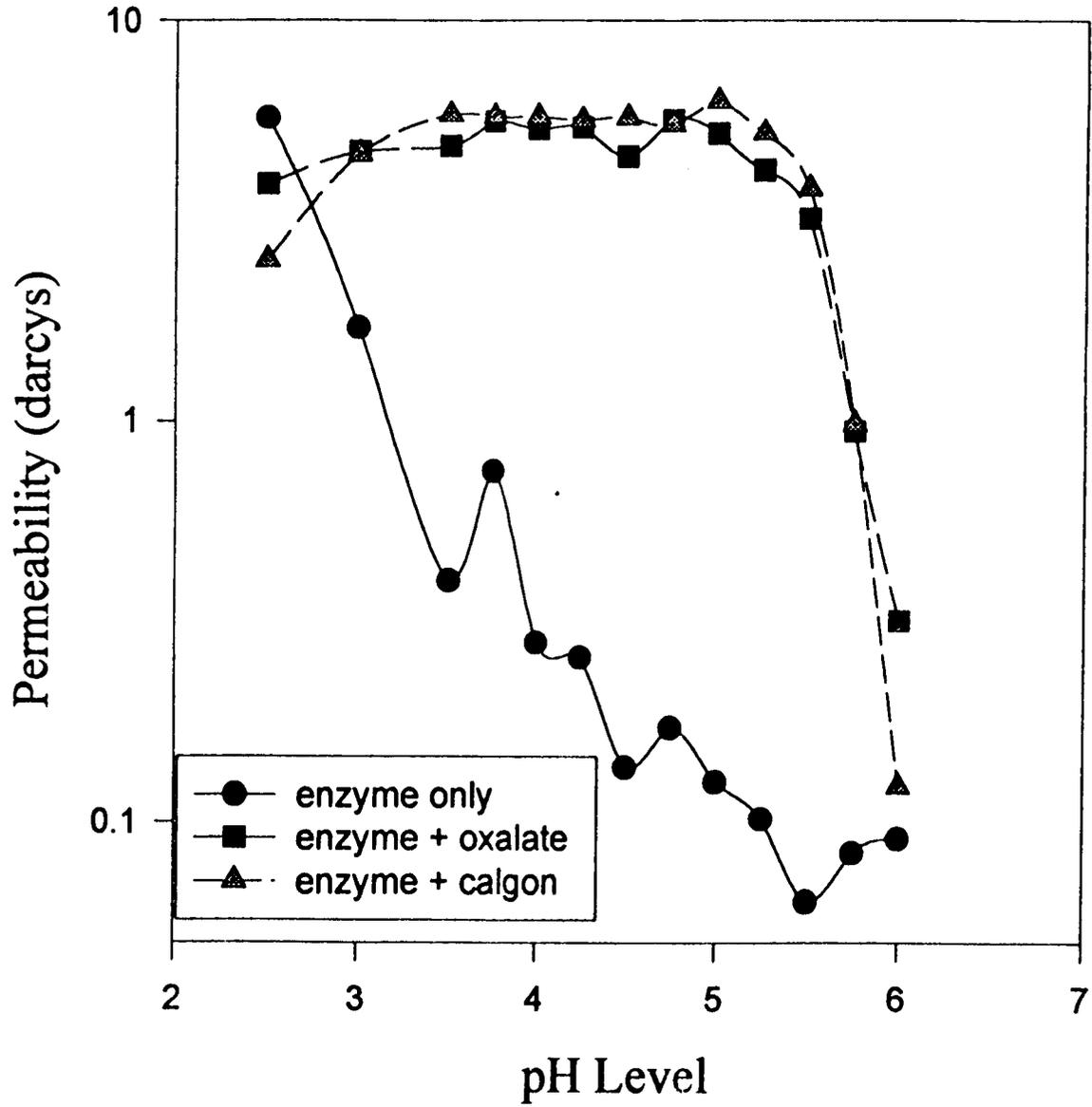


Figure 3.

Gas Permeability of Douglas Fir Cores



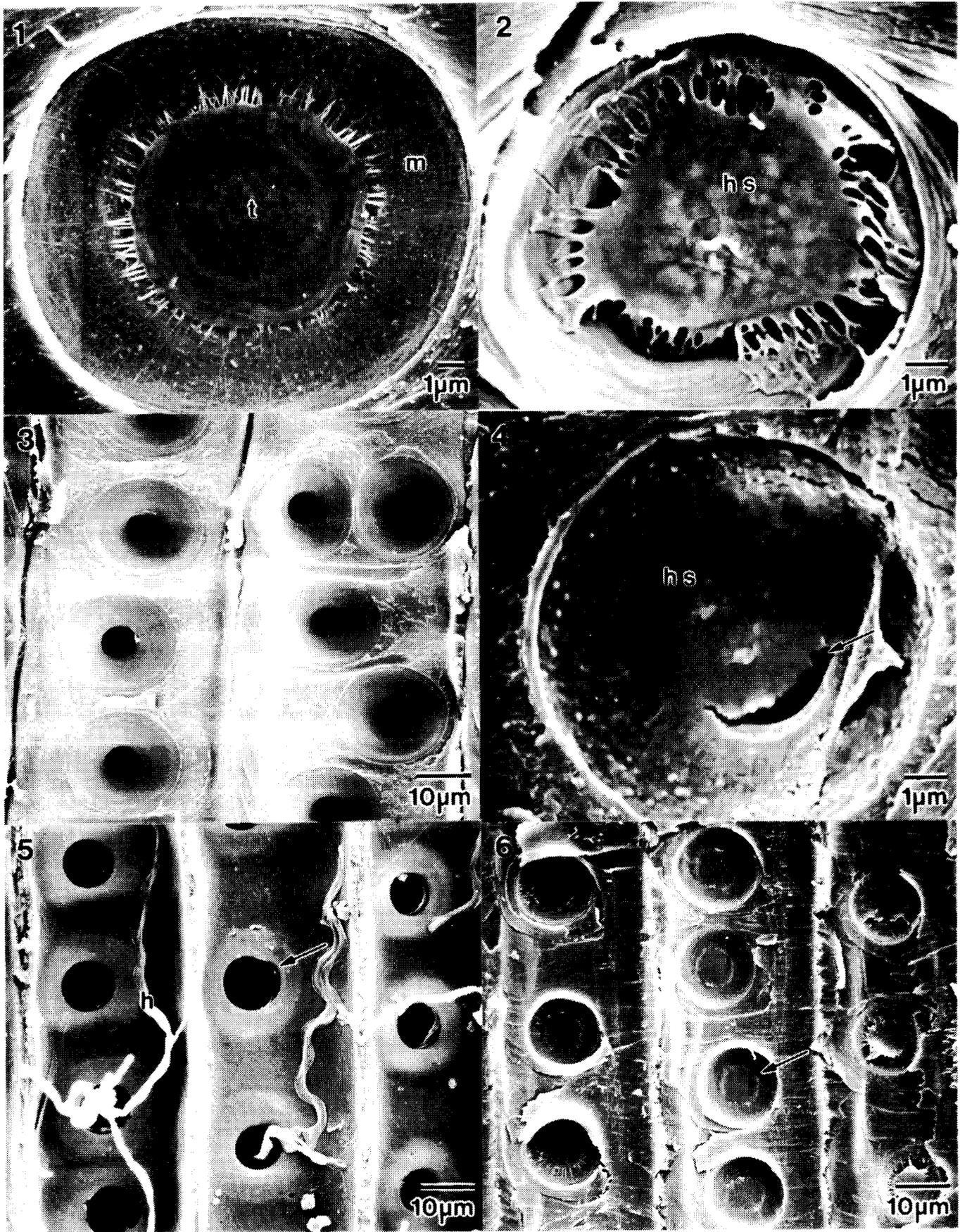


Figure 5--Scanning electron micrographs of Southern Pine bordered pits following brown-rot decay (14d):

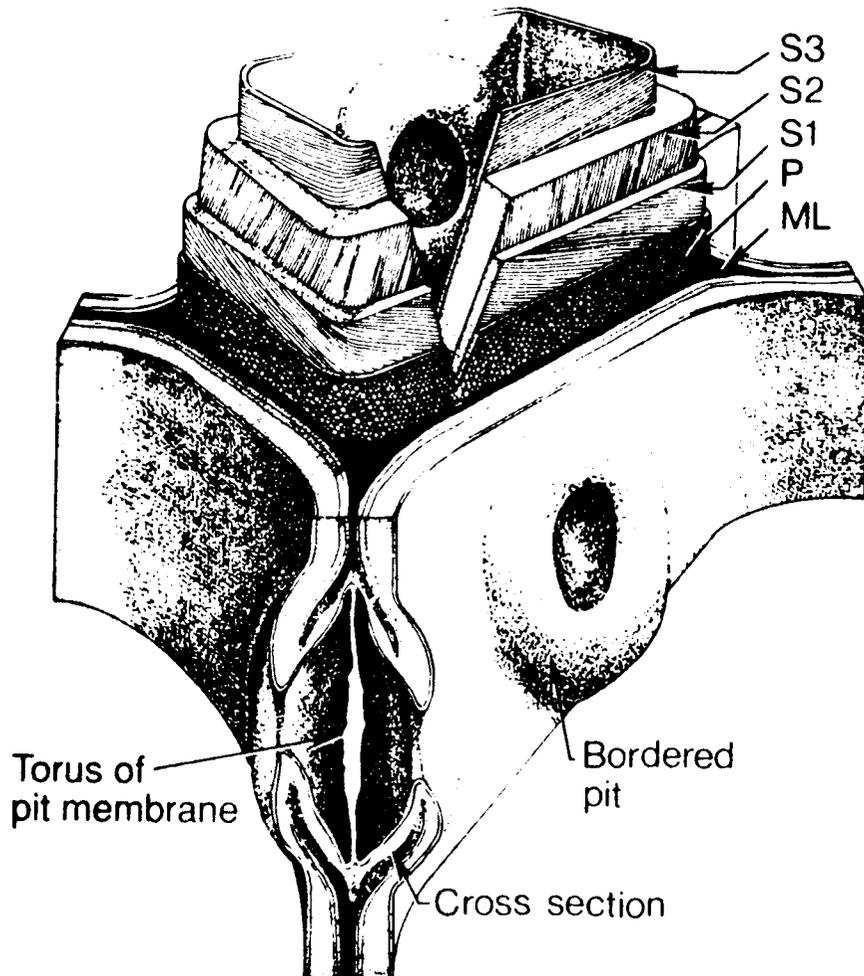


Figure 6--Bordered pit structure and its relationship to the layers of the wood cell wall of tracheids. Bordered pit membrane not aspirated.