PERMEABILITY CHANGES INDUCED IN THREE WESTERN CONIFERS BY SELECTIVE BACTERIAL INOCULATION

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ABSTRACT

The longitudinal gas permeability and impregnability with paraffin oil of western hemlock, grand fir, and Douglas-fir sapwood markedly increased after exposure to bacteria. Heartwood was not affected. *Bacillus polymyxa* was more effective in increasing gas permeability than any other single or mixed culture tested. Scanning electron microscopy suggested that this was largely due to degradation of bordered pit membranes.

Keywords: Tsuga heterophylla, Abies grandis, Pseudotsuga menziesii, permeability, bacteria, *Bacillus polymyxa,* sapwood, bordered pit membranes, torus.

INTRODUCTION

The permeability of wood—the extent to which it allows fluid flow—is critical in chemical impregnation processes whether for fire retardants, conventional creosote and pentachlorophenol preservatives, or reagents to chemically modify wood. Numerous methods have been tried to alter the structure of wood to increase its permeability. None have been widely adopted.

Several western conifers are commercially important species in which improved permeability would allow better preservative treatment: Western hemlock—one of the most important timber species in the Pacific Northwest—presents considerable difficulty in obtaining satisfactory sapwood treatment of round material (Dundas and White 1972) (even methods such as Boultonizing or steam conditioning are inefficient); the conclusions that various species of "white fir" (*Abies* spp.) do not treat easily or evenly and that incising should be required have been published by Blew and Davidson (1971); the refractory nature of Douglas-fir is common knowledge among wood treaters.

Various physico-mechanical and chemical pretreatments have been employed in efforts to improve permeability of these species, but virtually all of these have proved unsatisfactory. Biological pretreatments using fungi, bacteria, and isolated enzymes continue to receive a great deal of attention. Suolahti and Wallen (1958) and Ellwood and Ecklund (1959) were among the first to demonstrate that abnormally high permeability following wet storage of wood was due to bacteria. Knuth and McCoy (1962) proposed that *Bacillus polymyxa* was the major organism responsible for high porosity in ponded pine. Knuth (1964) later concluded that: many wood species are susceptible to attack by bacteria; both heartwood

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and sapwood may be degraded; bordered pit membranes, ray parenchyma cell contents and walls are broken down; and permeability can be increased appreciably. Bordered pit membranes are nearly always preferentially attacked, a phenomenon which has been linked to the pectolytic activity of several different species of bacteria.

Much of the research conducted since then has centered on the effects of water storage of several species—spruce in particular—and, to some extent, on the effects of bacterial isolates on permeability and/or fine structure of wood. Papers by Dunleavy and McQuire (1970), Bauch et al. (1970), Greaves (1970), Ward and Fogarty (1972), Fogarty (1973), Dunleavy et al. (1973), DeGroot and Sachs (1976) are representative of this research. A bibliography (Unligil 1969) and reviews of this topic have been written (Jutte 1971; Rossell et al. 1973).

This study was undertaken to determine whether single or mixed cultures of bacteria could bring about marked improvement in permeability of wood of three western conifers under controlled laboratory conditions with the thought that a biological pretreatment might be devised for improved preservative penetration.

MATERIALS AND METHODS

Short bolts were obtained from the bases of freshly felled trees, 8 to 10 inches DBH, of three species from the Pacific Northwest: Western hemlock (*Tsuga heterophylla*), grand fir (*Abies grandis*), and coastal Douglas-fir (*Pseudotsuga menziesii*). Immediately after cutting, the 2½-foot-long bolts were end-coated with emulsified asphalt, wrapped in polyethylene with paradichlorobenzene crystals, and air-freighted to the Forest Products Laboratory. Upon arrival, they were cut into disks approximately 3 inches thick, sealed in polyethylene, and stored at −29 C until used. Sapwood permeability cores were taken from the outer 1½ inches of a sapwood zone about 1¾ inches wide. Growth rate in this area was 8 to 10 rings per inch. Heartwood cores were removed from a maximum allowable distance from the pith.

Selection of organisms

Bacteria were selected on the basis of their known ability to degrade substrates of pectin, starch, cellulose, hemicellulose, or protein, or reported degradative effects on other wood species.

Most organisms tested were obtained from the Agricultural Research Service culture collection of the USDA, Peoria, Ill. Organisms and their culture designations were *Pseudomonas fluorescens* NRRL B-10, *Erwinia aroideae* NRRL B-138, *Cellulomonas biazotea* NRRL B-401, *Bacillus polymyxa* NRRL B-510Vat, *Xanthomonas campestris* NRRL B-1459A, *Azotobacter agilis* NRRL B-2270, and three Actinomycetes, *Micromonospora chalcea* NRRL B-1573, *Streptomyces globisporus* NRRL B-2872, and *S. longisporus* NRRL B-5336. Two additional bacterial cultures provided by James Ward of the Forest Products Laboratory were also tested: *B. cereus* and *E. nimipressuralis*.

For mixed cultures, organisms were selected that showed little or no antagonism in a paper disk compatibility test. This test consisted of spreading a pure culture of a test bacterium in liquid medium onto agar medium, dipping a sterile paper disk into a liquid culture of a second organism, and placing the disk onto the agar culture. After incubation, the mixed cultures were examined for zones of inhi-

bition around the disks, indicating a resistance of the two species to grow together. Organisms that produced a marked reduction in pH following their logarithmic growth phase were generally combined with those that showed a tendency to raise the pH of the media. Growth rates in pure culture were determined spectrophotometrically by measuring absorbence at 650 nm. Combinations were generally made using those organisms that showed a lag phase of similar duration before beginning logarithmic or exponential growth. Concurrent determinations of pH change and drop in growth rate measured by optical density supported the view that pH, rather than nutrient exhaustion, was in many cases responsible for a decline in growth rate.

Microtome sections

In the initial or screening phase, radial and tangential microtome sections were cut 25 μm thick from never-dried western hemlock sapwood and heartwood. Sections were immersed in 50 ml of one of three media and autoclaved for 20 min at 15 psi. Media used were nutrient broth, Dubos medium, and a salts medium consisting of 1.2% $NaNH_4HPO_4$, 0.6% Na_2HPO_4, 0.3% KH_2PO_4, 0.3% NaCl, 0.026% Na_2SO_4, and 0.001% $MgCl_2$. Some preparations contained 0.01 to 1% gluclose. All species listed in "Selection of Organisms" were used. Initially, incubations were for 3 days under either still or slow-rotary-shake conditions at 28 C. A 7-day incubation period was later adopted with only shake culture.

After incubation, the sections were removed from the medium, washed in distilled water, stained with safranin-O followed by picro-aniline blue, mounted, and examined with a light microscope. Autoclaving and presence of growth medium produced only minor changes in appearance of the microtome sections.

Permeability cores in nutrient media

Cylindrical cores 1.25 cm in diameter and 1.5 cm long were cut, some oriented with the long axis in the radial direction of the wood and some oriented longitudinally or parallel-to-grain, from never-dried sapwood of all three species. A set of individual cores was then submerged in nutrient broth with 1% glucose, autoclaved, inoculated (except for controls) with single or mixed cultures, and incubated for 54 days. A second set (one core per inoculum) was submerged in the broth with 0.5 N phosphate buffer added, and was incubated for only 42 days. The cores were then washed in water and slowly dried to equilibrium at 30% relative humidity. Incubation and drying were at 28 C.

Superficial nitrogen gas permeabilities were determined using apparatus described by Comstock (1968) except that a new specimen holder was devised by J. L. Tschernitz of the U.S. Forest Products Laboratory to circumvent the problem of the radial cores assuming an oval shape upon drying. Cores high in permeability were examined with light and scanning electron microscopes (SEM). Microtome sections for light microscopy were stained with safranin-O and picro-aniline blue. Specimens for SEM were coated with gold and examined at 20 kV with a Cambridge Stereoscan Mark II.

Permeability cores in soil bottles

This experiment was intended to determine whether large permeability increases could be effected by bacteria selected from previous experiments, but in the absence of appreciable nutrient medium.

To maintain the wood at a relatively constant moisture content under aerobic conditions without contamination, soil bottles were prepared according to ASTM D 1413 (1976) except that no feederstrip was used. Longitudinal permeability cores 1.25 cm in diameter and 3.8 cm long were placed in these soil bottles before autoclaving. In some cases, 10 ml of a nutrient solution consisting of 0.1% glucose and 0.1% sodium nitrate in distilled water was poured over the cores in the soil bottles, also before autoclaving. Inoculum, 1 ml from 24-hour nutrient broth shake cultures of either *B. polymyxa* or *X. campestris*, was placed on each core within the sterile soil bottles. They were then incubated for periods of 2 to 16 weeks at 28 C.

Initially, cores were removed after 4, 8, or 16 weeks of incubation and slowly dried to equilibrium at 30% relative humidity. Nitrogen gas permeability determinations were made. Measurements were also made of the percentage weight gain when the conditioned cores were evacuated for 20 min at 74 cm of mercury and then submerged in paraffin oil (U.S.P. No. 31) for 10 min at atmospheric pressure. In the final experiment, the only organism tested was *Bacillus polymyxa* because previous work had shown it to have the greatest effect on permeability. Replicates of five sapwood cores of each species were removed after each time interval, 0, 2, 4, 6, or 8 weeks' incubation. Heartwood cores were incubated for 8 weeks only. All cores were air-dried, end-coated with an epoxy-coal tar formulation, and tested for paraffin oil uptake. Some cores for SEM observations were immediately placed in ethanol as they were removed from the soil bottles and critical-point dried to minimize artifacts.

RESULTS AND DISCUSSION

Microtome sections

The technique of predicting permeability changes based on morphological changes produced in microtome sections when submerged in liquid cultures of bacteria was not particularly useful. Examination with the light microscope of tangential sections was satisfactory for detecting appreciable levels of degradation of the tori of bordered pit membranes, but changes in simple and half-bordered pit membranes were extremely difficult to discern. Considering that all three of these wood species possess considerable secondary thickening of ray parenchyma walls, it was not surprising that little, if any, degradation of these walls was apparent.

Because microorganisms in general, and bacteria in particular, tend to synthesize those catabolic enzymes that require a minimum of energy expenditure for substrate degradation, it was thought that a salts medium containing only the microtome sections of wood as a carbon energy source might result in greater morphological changes than when the nutrient media were used. This did not prove to be the case. The most pronounced degradation of bordered pit membrane tori occurred in shake cultures with nutrient broth and 0.1 to 1% supplemental glucose, but only in sapwood sections. Organisms having the greatest effect on these tori were: *C. biazotea*, *X. campestris*, *B. polymyxa*, *B. cereus*, and mixed cultures of *B. polymyxa* with *S. longisporos*, *A. agilis* with *X. campestris*, *C. biazotea* with *X. campestris*, *S. longisporos* with *S. globisporos* and *M. chalcea*, and *B. cereus* with *S. globisporos*.

TABLE 1. *Gas permeabilities of sapwood cores after 54 days incubation with single or mixed bacterial cultures in liquid medium.*

Wood species	Organism(s)	Permeability[a]	
		Longitudinal flow	Radial flow
		Darcys	Darcys
Western hemlock	*B. polymyxa*	3.94	0.110
	C. biazotea	0.28	0.059
	E. nimipressuralis	0.30	0.028
	S. longisporos and *M. chalcea* and *S. globisporos*	0.35	0.042
	A. agilis and *X. campestris*	0.69	0.020
	Control	0.44	0.076
Grand fir	*B. polymyxa*	6.41	0.079
	C. biazotea	0.73	0.041
	E. nimipressuralis	0.64	0.046
	S. longisporos and *M. chalcea* and *S. globisporos*	1.20	0.042
	P. fluorescens	0.50	0.029
	E. aroideae	1.00	0.058
	A. agilis and *X. campestris*	1.53	0.070
	Control	0.58	0.048
Douglas-fir	*B. polymyxa*	10.26	0.030
	C. biazotea	1.23	0.024
	E. nimipressuralis	0.96	0.054
	C. biazotea and *X. campestris*	1.08	0.020
	S. longisporos and *M. chalcea* and *S. globisporos*	0.49	0.030
	P. fluorescens	0.30	0.036
	E. aroideae	1.35	0.078
	A. agilis and *X. campestris*	1.22	0.040
	Control	0.62	0.049

[a] Superficial nitrogen gas permeability (steady state) in Darcys.

Permeability cores in nutrient media

Gas permeability values after incubation of the first set of cores for 54 days in shake cultures of nutrient broth with 1% glucose were determined for radial and longitudinal cores of all three wood species (Table 1). Sapwood cores only were incubated, separated by species, with the radial and longitudinal cores in the same container. Values are for single cores in each case. Changes in radial permeability were slight. Longitudinal permeabilities were, in several cases, increased appreciably; the greatest increase for each of the three woods was with pure cultures of *B. polymyxa*.

For the second set of cores (one per variable) submerged in the broth with the phosphate buffer and incubated for 42 days, changes in radial permeabilities were also negligible (Table 2). Longitudinal permeabilities, however, increased appreciably with a number of different pure and mixed cultures for all three wood species. Measurement of pH at the end of the incubation period showed that, in some cases, the buffer had been effective throughout in maintaining pH. There was no strong indication that addition of the buffer resulted in greater increases in permeability.

Again, *B. polymyxa* was highly effective as were mixed cultures of *C. biazotea*

TABLE 2. *Gas permeabilities of sapwood cores after 42 days incubation in buffered liquid medium.*

Wood species	Organism(s)	Permeability	
		Longitudinal flow	Radial flow
		Darcys	Darcys
Western hemlock	*C. biazotea* and *B. polymyxa*	5.23	0.038
	X. campestris and *B. cereus*	0.92	0.039
	B. polymyxa and *P. fluorescens*	0.52	0.034
	B. polymyxa and *E. nimipressuralis*	0.32	0.067
	A. agilis and *X. campestris*	0.63	0.070
	X. campestris and *C. biazotea*	4.78	0.040
	B. polymyxa	5.83	0.022
	C. biazotea	0.70	0.029
	Control	0.12	0.027
Grand fir	*C. biazotea* and *B. polymyxa*	8.33	0.045
	X. campestris and *B. cereus*	1.73	0.038
	B. polymyxa and *P. fluorescens*	2.78	0.051
	B. polymyxa and *E. nimipressuralis*	0.80	0.030
	A. agilis and *X. campestris*	0.66	0.035
	X. campestris and *C. biazotea*	10.01	0.041
	B. polymyxa	6.26	0.054
	C. biazotea	0.66	0.044
	Control	0.82	0.055
Douglas-fir	*C. biazotea* and *B. polymyxa*	9.17	0.048
	X. campestris and *B. cereus*	2.05	0.061
	B. polymyxa and *P. fluorescens*	7.58	0.024
	B. polymyxa and *E. nimipressuralis*	1.00	0.037
	A. agilis and *X. campestris*	3.11	0.043
	X. campestris and *C. biazotea*	6.23	0.267
	B. polymyxa	9.11	0.113
	C. biazotea	0.56	0.021
	Control	1.01	0.036

with *B. polymyxa* and *X. campestris* with *C. biazotea*. A comparison of control values in Tables 1 and 2 indicates the considerable variability encountered due to natural variation in the wood and experimental error common with this technique for assessing permeability. (This was one of the reasons for using oil uptake as a measure of permeability change in the final experiment.) Permeability differences of less than several-fold should be considered of little practical significance. As expected from the permeability values, examination of microtome sections from the permeability cores with large increases in permeability disclosed much degradation of longitudinal tracheid bordered pit membranes, but little, if any, change in ray tissue.

Permeability cores in soil bottles

Cores incubated in bottles provided gas permeability and oil uptake values (Table 3) and specimens for SEM examinations.

Moisture content of the cores during the 4- to 16-week incubation periods was not appreciably altered from initial (green) condition. With each wood species, gas permeabilities and paraffin oil uptake were appreciably higher than with the

TABLE 3. *Gas permeability and oil percentage weight gain in sapwood cores incubated in soil bottles.*

Wood species	Organism	Nutrient	Incubation period	Permeability[a]	Oil-weight gain
			Weeks	Darcys	%
Western hemlock	*X. campestris*	Yes	4	0.13	
			8	0.21	
			16	0.22	
		No	4	0.29	
			8	0.30	
			16	0.23	
	B. polymyxa	Yes	4	6.16	
			8	5.91	
			16	8.40	98
		No	4	4.82	
			8	7.62	
			16	8.23	
	Control	Yes	4	0.31	
			8	0.21	37
			16	0.28	
		No	4	0.24	
			8	0.27	
			16	0.54	
Grand fir	*X. campestris*	Yes	4	0.75	
			8	1.28	
			16	0.44	
		No	4	1.27	
			8	0.25	
			16	2.19	
	B. polymyxa	Yes	4	15.3	
			8	14.4	
			16	13.8	
		No	4	9.78	
			8	12.3	
			16	14.0	118
	Control	Yes	4	0.51	
			8	1.15	
			16	0.48	
		No	4	1.28	
			8	1.62	70
			16	0.85	
Douglas-fir	*X. campestris*	Yes	4	0.76	
			8	1.17	
			16	0.53	
		No	4	0.37	
			8	0.30	
			16	0.23	
	B. polymyxa	Yes	4	9.10	
			8	11.1	
			16	10.2	91

TABLE 3. *Continued.*

Wood species	Organism	Nutrient	Incubation period	Permeability[a]	Oil-weight gain
			Weeks	Darcys	%
		No	4	3.34	
			8	8.81	
			16	10.8	
	Control	Yes	4	0.26	
			8	0.18	
			16	0.33	
		No	4	0.18	
			8	0.26	
			16	0.38	55

[a] Longitudinal, superficial nitrogen gas permeability (steady state).

uninoculated control. The supplemental nutrient had no pronounced effect on permeability. *X. campestris* did not produce any marked change in gas permeability.

A rather cursory examination of a few of these air-dried cores was made with SEM. Western hemlock cores inoculated with *B. polymyxa* showed extensive degradation of the tori of bordered pit membranes. After 16 weeks' incubation, most tori were missing with the margo barely discernible in the pit chamber. A number of bordered pits were observed with the torus in a partially degraded condition. In the grand fir specimens, following 4 weeks' incubation with *B. polymyxa*, virtually all bordered pits were open. Many simple and half-bordered pit membranes exhibited some degree of degradation attributable to bacterial action. Douglas-fir, incubated for 16 weeks with *B. polymyxa*, showed very few intact bordered pit membranes.

From the low permeability values obtained with all three wood species following incubation with *X. campestris*, one would expect little degradation of bordered pit membranes connecting longitudinal tracheids. This expectation was confirmed with the scanning electron microscope which showed numerous pits with bacteria present on the torus, but the membrane intact and tightly aspirated (Fig. 1).

The objective of the final experiment, in which cores were removed and dried at intervals, was to determine the length of the incubation period necessary to effect permeability increases and to obtain a statistically significant measure of those increases. Table 4 presents mean percentage weight gain of paraffin oil for five replicates for each species and incubation period. Lines are drawn under those groups of means that are homogeneous (i.e., no statistically significant difference) at a 95% confidence level. Clearly, most of the permeability increase occurred in the first 2 weeks. However, except for grand fir, the permeability as measured by oil uptake was significantly greater after 8 weeks than 2 weeks. The data in Table 4 are all for sapwood cores.

A single heartwood core of each species was incubated for 8 weeks. Oil uptake or absorption of these cores was virtually identical to that of the uninoculated

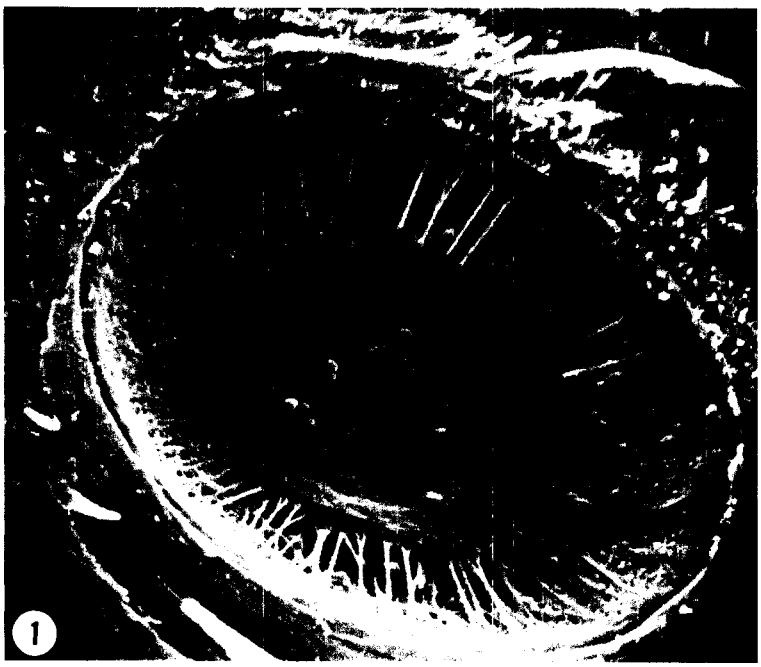

FIG. 1. Bordered pit of western hemlock supporting *Xanthomonas campestris* bacteria, but showing no degradation of the pit membrane after 16 weeks' incubation (6,000×).

heartwood controls. Interestingly, the heartwood samples absorbed approximately the same amount of oil as did the sapwood controls. Western hemlock heartwood showed a 39% weight gain compared with 35% for the sapwood control. Grand fir showed a 46% oil weight gain in comparison with 40% for the sapwood. The Douglas-fir heartwood sample showed a 33% oil weight gain as did the sapwood control.

As mentioned in "Materials and Methods," cores from this experiment were removed after each of the four incubation periods and critical-point dried to maintain the bordered pit membranes in a nonaspirated condition with a minimum of artifacts and examined with SEM. In a grand fir bordered pit membrane not

TABLE 4. *Paraffin oil uptake of sapwood cores incubated in soil bottles with* B. polymyxa.

Species	Oil uptake[a] (weight gain)				
	Incubation period (weeks)				
	0	2	4	6	8
	%	%	%	%	%
Western hemlock	35.0	67.0	84.8	102.4	122.0
Grand fir	40.2	126.4	127.4	133.4	137.2
Douglas-fir	33.4	78.4	103.4	102.6	120.20

[a] Mean of five replicates. Underlined values are *not* significantly different from one another at a 95% confidence level.

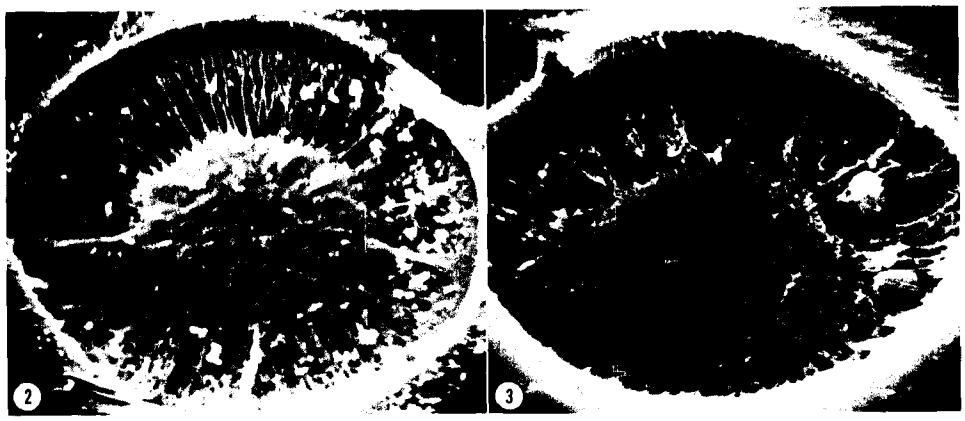

FIG. 2. Bordered pit membrane in the sapwood of grand fir. The central torus appears to be quite amorphous in nature (4,000×).

FIG. 3. A grand fir bordered pit membrane after 2 weeks' incubation with *B. polymyxa*. The torus no longer appears amorphous, but rather exhibits a nonoriented microfibrillar structure. Two bacterial cells are contained in the pit chamber below the margo (4,000×).

exposed to bacteria, the torus is apparently coated with an amorphous substance (Fig. 2). After 2 weeks of incubation, the amorphous material appears to have been degraded by bacteria leaving a nonoriented microfibrillar structure and fine openings in the torus (Fig. 3). Some degradation of the margo also seems to have taken place. With 6 weeks of incubation, most bordered pits were completely open. The work of Bauch and Berndt (1973) suggests that this amorphous substance is largely pectin. Its degradation by *Bacillus* is in agreement with reported pectinase activity (Fogarty and Ward 1973). Appearance of the tori after exposure to *B. polymyxa* is similar to micrographs of pit membranes exposed to isolated pectinase published by Nicholas and Thomas (1968), Tschernitz (1973), Meyer (1974), and Tschernitz and Sachs (1975).

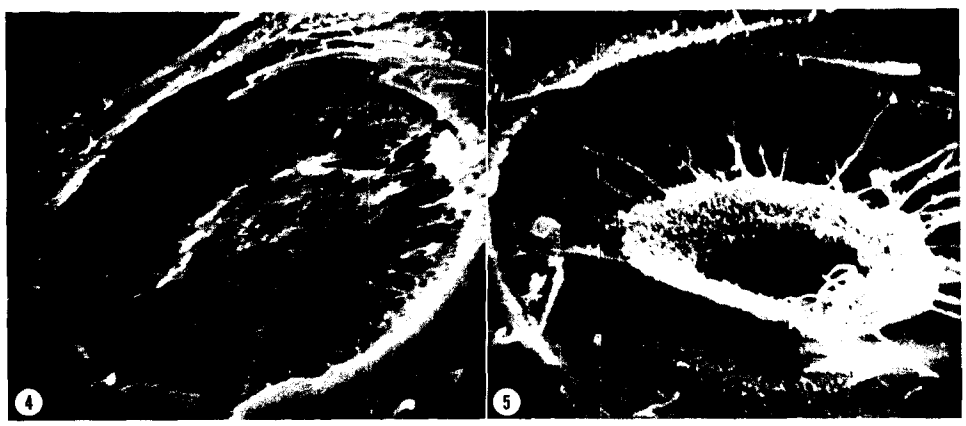

FIG. 4. Bordered pit membrane in the sapwood of Douglas-fir. The surface of the torus appears quite amorphous (4,000×).

FIG. 5. Douglas-fir membrane after 8 weeks' incubation with *B. polymyxa* (4,000×).