

AN ABSTRACT OF THE THESIS OF

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Title DETECTION, IDENTIFICATION AND CONTROL  
OF PORIA CARBONICA AND OTHER FUNGI  
IN DOUGLAS FIR POLES

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Abstract approved \_\_\_\_\_  
(Major professor)

Internal deterioration by Poria carbonica of Douglas-fir poles, used for electric power transmission and distribution, occurs frequently in the Pacific Northwest. This condition requires the untimely replacement of the poles. Fungal infection in its early stages, prior to the occurrence of visible damage, was detected by sampling poles in the more frequently moist and aerated ground line zone. An increment borer was used for the extraction of wood cores, which were then cultured on malt extract agar for two weeks at room temperature. Pure fungal cultures prevailed in instances of incipient decay while mixed microbial populations developed from cores taken in the vicinity of advanced decay pockets. Of the visible damage-free wood cores cultured, more than 50 percent yielded live fungi. Rapid evaluation of the wood destroying ability of the isolates was obtained by the examination of thin sections from the wood cores incubated

for detection purposes. Under those conditions, P. carbonica produced numerous bore holes in the tracheid walls. More specific identification was provided by the fluorescent antibody technique.

Control of P. carbonica in poles in service required the development of new approaches to pole treatment. The following hypotheses were explored with encouraging results:

1. Fungicidal gases, singly or mixed, can penetrate throughout poles at room temperature under atmospheric pressure, destroy P. carbonica, and provide residual action.

2. Live microbial commensals within the host can provide protection against the invading pathogens.

For experimental verification, methyl bromide and ammonia in the presence of atmospheric oxygen were selected. This halide gas performed singly as conceived; it also reacted with ammonia, yielding a residual ammonium bromide salt. For the second hypothesis, a Douglas-fir pole heartwood commensal, tentatively designated as F-y, was isolated. It was found to kill P. carbonica on malt extract plates. No live P. carbonica was recovered from pole sections permeated with F-y as a result of natural or artificial inoculation. No damage to tracheid wall was observed at the systemic or cellular levels in F-y pervaded pole sections.

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DETECTION, IDENTIFICATION AND CONTROL OF  
PORIA CARBONICA AND OTHER FUNGI  
IN DOUGLAS FIR POLES

by

JACQUES LOUIS RICARD

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Date thesis is presented May 10, 1966

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DETECTION, IDENTIFICATION AND CONTROL OF  
PORIA CARBONICA AND OTHER FUNGI  
IN DOUGLAS FIR POLES

PREFACE

Douglas-fir poles are used extensively for electric power transmission and distribution throughout the Northwest. Their number in the state of Oregon is estimated at over one million.

Although the life span of a properly treated pole could approach fifty years, exposure of untreated wood because of checking beyond the treated shell may result in extensive heartwood deterioration within eight years of the placement of a pole in service. Replacement cost of a pole varies from \$300. to over \$1,000., depending upon size and location.

The Oregon State University Forest Research Laboratory in cooperation with Bonneville Power Administration embarked on a comprehensive research program for the detection of decaying poles and treatments to prevent further deterioration. During the course of this research a basidiomycete, Poria carbonica, was shown to be chiefly responsible for the heartwood deterioration of Douglas-fir poles.

Another aspect has been the exploration of potential contributions of microbiological techniques to the problem. This particular endeavor, which is described here, includes three major sections:

(1) detection of fungal infection before visible deterioration has occurred, (2) identification of fungi detected, and (3) control of wood-destroying microorganisms within the poles in service, with the objective of destroying the decay fungi and, if possible, preventing reinfestation.

The scope of the study was such that only the deductive approach appeared practical. The experiments were used to test the validity of the hypotheses developed and positive results are believed to have heuristic value, rather than conclusive meaning. In every area explored, further work is in progress to ascertain more accurately the limitations of the techniques used.

Two descriptive expressions used throughout the following pages should be qualified: Poria carbonica was not formally identified in every case for obvious reasons. It is known to have a rather distinct mycelium and also to be the predominant heartwood destroyer in Douglas-fir (Mothershead and Graham, 1965). Whenever typical mycelium was observed in cultures it was assumed to represent P. carbonica. This mycelium is described by Lombard and Gilbertson (1965) as: "white, soft, felty to woolly mat, medium growth, negative oxidase reactions, and presence microscopically of chlamydospores, conidia, and stiffly branching hyphae." Further study may, in certain cases, require allocation to another species. A more complete description of the organism is reported in the Appendix. The

other coined expression: F-y represents a Deuteromycete with a whitish grey, soft, partially embedded mycelium, exhibiting moderate growth. A bright yellow water soluble pigment is produced by recent isolates or cultures growing in a competitive environment. Light brown to black conidia occur in chains on hyphal tips after the mycelium is at least two weeks old. Old cultures appear entirely black from the development of conidia over the whole surface. The shape of these asexual spores ranges from round to oval.

## SECTION I. DETECTION

### INTRODUCTION

Fungal decay in poles ranges from incipient decay, where the fungus is present but no directly visible damage has yet occurred, to advanced decay, where directly visible damage is present. This latter stage varies from a few concentric checks of the heartwood with an occasional accumulation of white fungal mycelium appearing in the voids to actual empty spaces in the heartwood or decay pockets.

Advanced decay can be detected in a number of ways, as reported by Mothershead and Graham (1962) using radiographic, ultrasonic and electric resistance measurements.

Incipient decay is the only form of fungal infection considered here. A convenient technique for that purpose is essential to evaluate significant microbial populations in poles: new poles prior to conventional preservative treatment, poles in service, infected poles before and after treatment. New poles had to be tested in order to ascertain when contamination occurred, whether in the forest prior to cutting, in the storage yards or upon placement of the treated poles in service. Routine inspection of poles in service needed some techniques to identify essentially intact poles, but subject to deterioration unless appropriate treatment is applied. Experiments on control could provide meaningful results only if the microbial population was tested accurately.

## REVIEW OF LITERATURE

Numerous techniques have been described for the culturing in the laboratory of pole or other wood samples removed from a wood structure. Wilcox (1964) relates the isolation of fungi from wood as follows:

Methods for the isolation of microorganisms from plant tissues were discussed by Riker and Riker (1936), and the procedure for isolation of fungi from wood was described by Cartwright and Findlay (1958). Since the surfaces of wood specimens may be contaminated with spores of fungi capable of growing more rapidly than the wood-destroying Basidiomycetes, it is necessary to . . . remove the surface layers before transferring tissue to a nutrient medium. Very small pieces of wood from the interior of the specimen should be transferred to minimize the chances of obtaining more than one organism. Since wood-inhibiting organisms are sometimes irregularly distributed within wood, it is necessary to transfer from several areas within a given sample. A chisel forceps and other special equipment designed to facilitate the isolation of fungi from wood were described by Hubert (1929), but a flamed scalpel is also satisfactory.

An isolating tool also has been designed by Dr. W. F. Eslyn of the Forest Products Laboratory. It consists of a small-bore leather punch mounted on a wood handle. The punch is fitted with a rod which may be pushed through the bore of the instrument to extract the small cylindrical plug which is formed. A rapid isolation method was suggested by Scheffer (1965) for isolation from wood which is not appreciably contaminated with molds or other rapidly growing organisms. By this method a wood sample is sawn into small blocks. Each block is speared with a dissecting needle at one corner and rotated quickly in a flame to surface-sterilize it. The block is then placed on a nutrient medium with the needle.

Opsal (1964) uses a disinfected knife or scalpel for field sampling, limiting the area tested to the surface of the wood.

Wilcox (1964) goes on with a discussion of the culture medium:

Wood decay fungi are commonly cultured on an agar medium containing two percent malt extract. Although this medium appears to be quite satisfactory, other possible culture media have been discussed by Cartwright and Findlay (1958) and by Riker and Riker (1936).

None of these methods are very convenient for large scale field application, as Mothershead and Graham (1962) pointed out:

In general, laboratory methods for detection of decay are complex, time-consuming, and require exact conditions and careful observations . . . . field tests for detection of decay are more difficult to devise in many ways than laboratory tests, in that they must be simple to apply, accurate in appraisal, and, most difficult of all, rapid in evaluation.

These authors (1962), however, indicated in a different section of the same publication that "where internal decay may be indicated by sounding or visual evidence, size and location of the pocket is determined by increment borings or with a drill with a long bit." This last procedure together with the established culture techniques provided a basis for the development of an incipient decay detection method.

Since the detection method described here was developed, Greaves and Savory (1965) reported an almost identical one, the only appreciable difference is in the handling of the sampled wood: "all moist material was dried in the laboratory for two days prior to making isolations. This effectively eliminated major bacterial growth on the isolation media which would have swamped fungal development." No such elimination was found necessary here.

## MATERIALS AND METHODS

The procedure devised for the detection of incipient decay combines essentially the increment borer sampling techniques--using necessary precautions to avoid contamination--with the laboratory culture on malt extract agar medium, thereby achieving a test of the pole in depth for heartwood decay organisms. The original sampling kit, Figure 1, and two typical plates after two weeks in incubation, Figure 2, are shown on page 9.

### Culture Media

Media different in composition and also in malt extract concentration were tested with a known culture of Poria carbonica before selection of the formula used routinely.

Two test cultures were provided for this purpose by O. F. Hand, Materials Testing Laboratory, Bonneville Power Administration, Vancouver, Washington. These cultures were labeled M89 and 91, respectively.

The media tested included Czapeck agar, peptone dextrose agar, tannic acid agar and two media (I and II) modified from Fukuzumi's formula (1960). These media were prepared as follows:

<u>Medium I</u>	<u>Grams</u>
wood meal Douglas-fir $\cong 4\mu$ average diameter	1
agar	15
xylose	5
asparagine	2.5
yeast extract	2.0
distilled water	1 liter
pH adjusted to 6.0	

<u>Medium II</u>	<u>Grams</u>
wood meal Douglas-fir $\cong 4\mu$ average diameter	25
peptone	0.4
$K_2HPO_4$	0.6
$MgSO_4 \cdot 7H_2O$	0.2
$CaCO_3$	0.4
thiamine HCl	.002
distilled water	1 liter

Three concentrations of malt extract were used, those recommended by Nobles (1948), Alexopoulos (1962), and in the Difco manual (1963):

	<u>Nobles</u>	<u>Difco</u>	<u>Alexopoulos</u>
Malt extract	12.50	30	25
Agar	20	15	15
Distilled Water	1 liter	1 liter	1 liter



Figure 1. Original sampling kit used for the detection of incipient decay. The glass tube and metal oil can were replaced eventually with regular and squeeze plastic bottles respectively.



Figure 2. Typical pole cores incubated on malt extract agar for three weeks. The fungi free plate indicates a sound pole; the mixed fungal population in the other plate reflects advanced decay in the vicinity of the sampling point.

The concentration suggested by Alexopoulos appeared best suited for the purpose in terms of fungal growth rate and abundance.

### Sampling Procedure

The sampling procedures for routine testing is described as follows (Ricard and Mothershead, 1966):

1. Swab surface of pole at point of sampling with 95 percent ethyl alcohol on a cotton wad to remove surface contaminants.
2. With an appropriately sized leather punch that has been dipped in alcohol, then shaken vigorously or ignited, remove a wood plug about 1/4 inch long for further protection from contamination and to facilitate starting the borer. The punch and other tools are shown in Figure 1.
3. Using an alcohol-treated increment borer, obtain a core from six to eight inches long.
4. Remove the core with an alcohol-treated extractor and transfer it rapidly to a sterile tube. Several screw-cap glass or disposable plastic varieties are available.
5. Flood the hole left in the pole with preservative, then drive in a treated wood plug.
6. Upon delivery to the laboratory, embed the cores in sterile malt agar prepared according to Alexopoulos' formula (1962) or some other formulation acceptable for growth of wood-destroying fungi.

The plates are then incubated for two weeks at room temperature.

## RESULTS

No medium produced results superior to malt extract in terms of growth rate, although Medium I and II were equally suitable, Figure 3. Fukuzumi's medium has the advantage of a more precise composition, but this feature is not as valuable for routine detection cultures as for physiological studies. Malt agar was therefore preferred because of its simpler preparation procedure. The various concentrations of malt extract tested indicated an optimum for growth rate at the level suggested by Alexopoulos (1962).

The other media tested, peptone dextrose agar, tannic acid agar, and Czapeck agar, provided less rapid and less profuse growth in the order listed.

### Trial Cultures

The detection technique was next tested on several pole sections stored at the laboratory. These sections showed various levels of visible decay ranging from slight to obvious decay pockets.

The live fungal population was found to vary appreciably with the condition of the pole, Figure 4.

Cultured increment borer cores from apparently sound pole sections produced no live microorganisms in some cases and Poria carbonica in others. Frequently, pole sections showing advanced



Figure 3. Three week old Poria carbonica on Medium I; wood meal, xylose, asparagine, yeast extract, agar and water.

A. No visible deterioration, i. e. incipient decay; the samples yielded only Poria carbonica.

B. Visible deterioration, i. e. advanced decay; the samples yielded a mixture of fungi even several cm away from the decay pockets.

Figure 4. Cultures on malt extract agar obtained from pole sections at various stages of decay



decay yielded no Poria carbonica although a number of different fungal colonies would appear. The results of the preliminary tests in the laboratory were reported (Ricard, 1964) as follows:

The reliability of the sampling and culture technique was evaluated with the sampling of four pole sections kept in the laboratory: three decayed sections ranging from incipient to advanced stages, and one sound section. Figure 4 shows the cultures obtained and the corresponding sections sampled. The sound wood yielded no growth; the slightly decayed wood revealed only one type of invader (Figure 4A), while a varied microbial population was obtained from the heavily decayed section (Figure 4B).

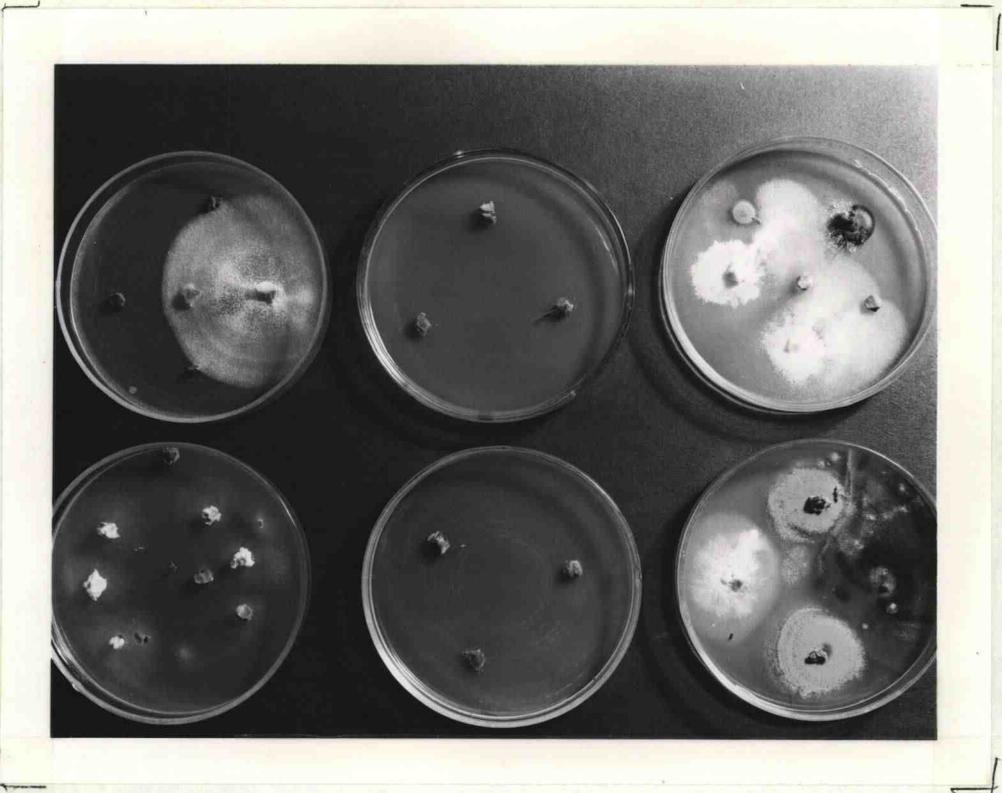
Plates were examined frequently as incubation proceeded and the observations made are recorded in Table I. Plates after three weeks incubation at room temperature are shown in Figure 5. Because no new colonies developed from the wood samples after one week, this time period was selected as the minimum incubation period for routine cultures. Note in Table I that the only wood samples which did not yield fungi were extensively decayed. By the time visual evidence of decay has developed, the fungi may be autolyzed. The opposite extreme, sound wood, also lacks live fungi, Figure 6.

Table I. Rate of development of fungi on malt extract agar plates incubated at room temperature ( $80^{\circ}\text{F} \pm 20^{\circ}\text{F}$ )

Sample No.	Pole identification	Appearance of wood samples	First observation of hyphae in days of incubation	Last observation of new colonies in days of incubation
1	ALB-18	sound	6	6
2	ALB-18	sound	6	7
3	ALB-18	early decay	6	6
4	M-6	advanced decay	None	None
5	M-6	sound	7	7
6	M-6	early decay	4	5
7	G-1028	sound	5	6
8	G-1028	early decay	4	5
9	G-1028	advanced decay	4	4

Figure 5. Three week old fungal populations on malt extract agar from pole samples: pure culture on left, mixed population on right. These samples were obtained from poles with no visible decay in one case, plate on left; extreme decay pockets in the other, plate on right.

Figure 6. Microbial population from three different poles, from left to right. The top and bottom plates are replicates from the same pole. Note the middle plates: no microbial growth, i.e. sound pole.



### Field Tests

The technique was then applied in the field: seven new and three used poles ranging from ten to eighteen years of age since treatment were sampled at Consumers' Power yard in Corvallis. These poles showed no visible decay and none yielded any wood destroyers when cultured.

Forty-nine poles were then sampled by Mr. J. S. Mothershead (Mothershead and Graham, 1965); of these, fourteen were found free from visible decay, yet decay fungi grew from the cultured cores. The same incidence prevailed in about twenty poles tested by the writer for the identification of suitable test poles for chemical control experiments.

The source of infection was next considered because there was no reliable evidence to indicate whether or not the infection originated in the forest prior to the original preservative treatment or after installation of the treated poles in service.

Over 100 new poles were sampled at three different treatment plants with the cooperation of Mr. A. T. Bode, Bode Inspection, Inc. and Mr. Del Cooley of the McCormick and Baxter Creosoting Company in Portland, Oregon, then cultured at the Laboratory. In a few instances, punky knots pointed out to the occurrence of fungal infection extending deep into the wood and usually accompanied by staining

as shown in Figure 7. These poles were rejected by the inspectors as usual. None of the new poles passed by the inspectors yielded wood decay fungi.

It was concluded that initial inoculation of the poles takes place after treatment usually. In the opinion of Mothershead and Graham (1965) fungal infection results from the checking that exposes untreated wood to contamination.

The second relatively large sampling took place in the yard of Consumers Power, Inc., Corvallis through the cooperation of Mr. L. J. Stubkjaer, used poles taken out of service for reason other than decay were sampled and cultured. Results are summarized in Tables II and III.

All pole samples cultured were free from visible decay. Neither age nor size of pole appear to be highly correlated with infection. These data indicate that infection may start as early as four years and be absent as late as nineteen years after purchase of a pole.

The thirty-three poles, where Poria carbonica was found, have been discarded as a result of the findings in the cultured samples, while the thirty-five poles free from infection were dipped in preservative solution and returned to service. The thirty-five poles where fungi other than Poria carbonica occurred have been held for further sampling.

This last set of samples revealed an interesting event: at least

ten poles contained incipient infection with a pure culture of a fungus that produced colonies different from Poria carbonica and secreted a yellow water soluble pigment. This organism, described as "F-y" in further discussion, was readily noticed by the bright yellow appearance which it imparted often to the entire culture substrate.

A number of other poles were tested with this procedure in various surveys for the determination of incipient decay incidence or for the evaluation of control treatments. At least one utility company plans to use this procedure for routine inspection of poles in service, starting this year.

Table II. Incidence of decay in relation of height of used Douglas-fir poles

Height in ft	Total		Sound		Questionable		Infected	
	Actual	Percent	Actual	Percent	Actual	Percent	Actual	Percent
30	23	100	6	26.1	10	43.5	7	30.4
35	31	100	5	16.0	13	42.0	13	42.0
40	21	100	11	52.4	6	28.6	4	19.0
45	13	100	6	46.1	4	30.8	3	23.1
50	9	100	7	78.0	-	-	2	22.0
55	4	100	-	-	1	25.0	3	75.0
60	1	100	-	-	-	-	1	100
65	1	100	1	100	-	-	-	-
TOTALS:	103		36		34		33	

Table III. Incidence of decay among used poles in relation to age group since purchase

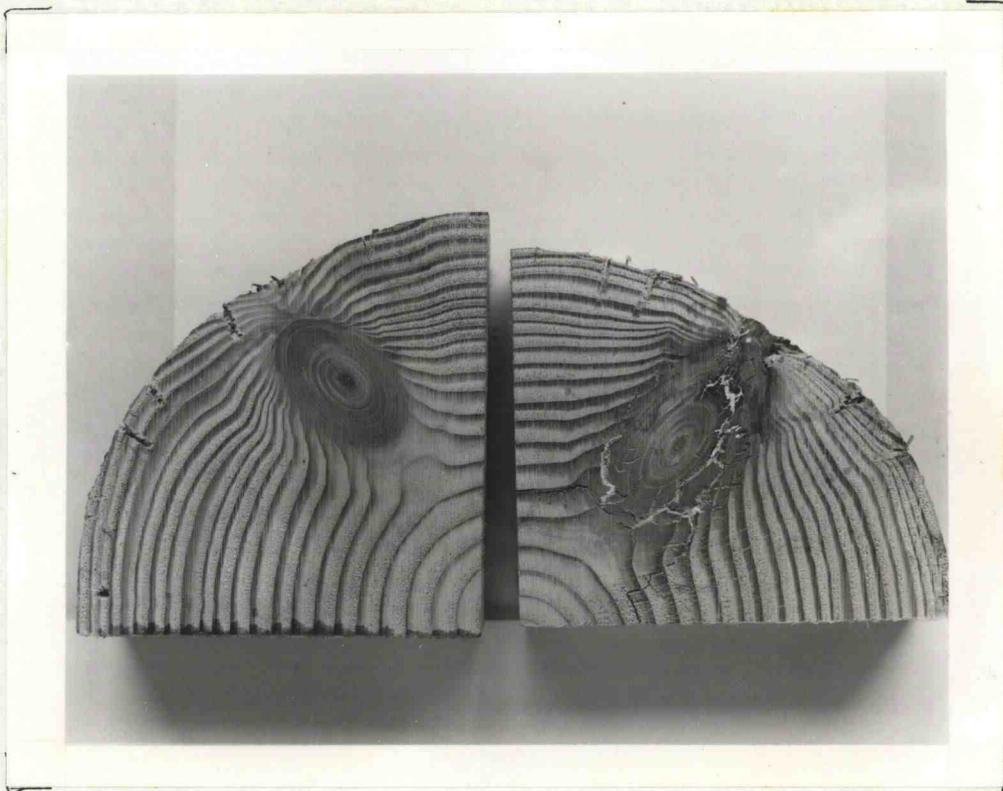
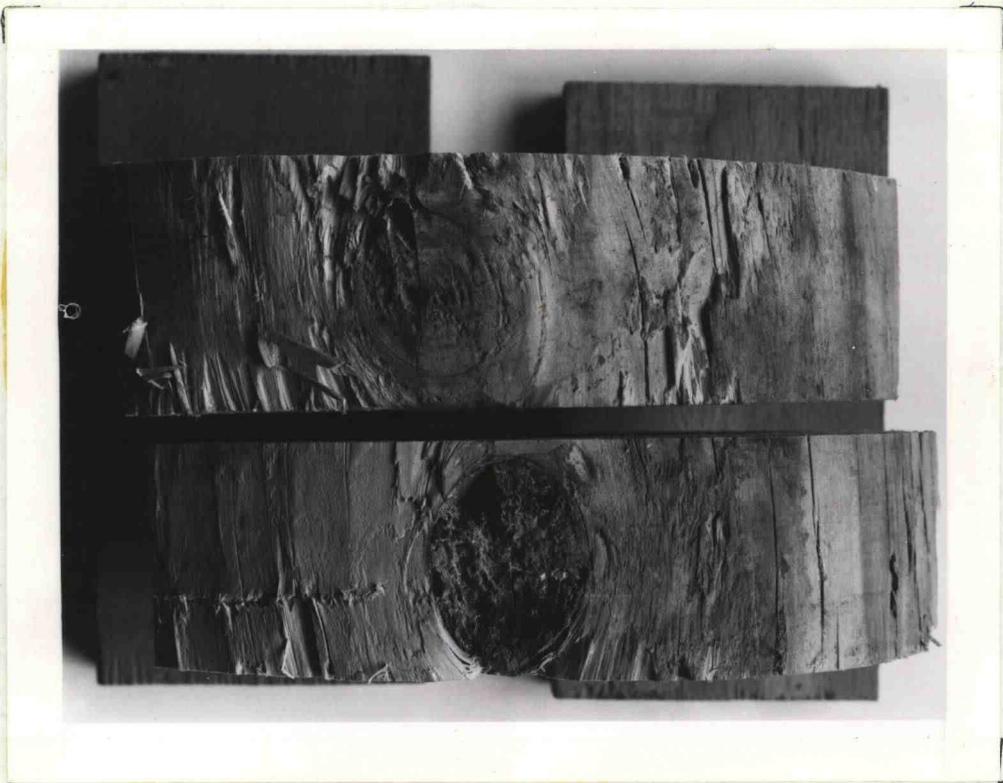
Age	Total		Sound		Questionable*		Infected	
	Actual	Percent	Actual	Percent	Actual	Percent	Actual	Percent
0-4	9	100	7	78	1	11	1	11
5-9	8	100	3	37	4	50	1	13
10-14	16	100	6	37	3	19	7	44
15 and over	38	100	12	35	16	39	10	26
Unknown	32	-	7	-	11	-	14	-
TOTALS	103	-	35	-	35	-	33	-

\*These poles contain live fungi other than Poria carbonica. Since these cores were taken the current detection procedure was modified to reduce the number of poles in this group: if an atypical fungus develops from the core upon plating a thin section is prepared, upon completion of incubation, and examined for bore holes. If some are observed the pole is classified as infected, see identification section for further details and illustrations.

A. The top knot is normal, the bottom one is punky or soft.

B. White, live fungal mycelium is visible around the punky knot, on the right; the knot on the left is normal.

Figure 7. Fungal infection in a new pole viewed from the outside A, and in a radial section B.



## DISCUSSION

The culturing of field samples is intended primarily as a qualitative test, to indicate whether or not a given pole is free from significant microbial infection.

The exact probability of infection detection as a function of the number and size of cores sampled has not been computed. Obviously a surface sampling of the pole is not adequate to determine heartwood infection. The core removed for culturing must include material from the entire radius of the pole, excluding the portion permeated with preservative. The boring should be taken in the groundline zone where the moisture is most likely to fall within the range suitable for fungal growth. For this reason as well as on the assumption that the wood destroyers enter the heartwood through seasoning checks exposing untreated wood, the core was usually taken immediately below checks ending within one foot above the groundline; if the check extended below that level the core was taken within six inches on the side of the check. A greater number of samples within the groundline zone would undoubtedly increase the probability of detection, but whether the increased certainty would be commensurate with the increased effort remains to be determined.

In the various tests described, only one sample per pole was taken. On a few occasions, poles with decay pockets were not

detected by visual examination of borings, but subsequent culture tests indicated that the poles were infected, and decay pockets were found where the poles were crosscut. No instance of a sterile core from an infected pole has been recorded. Admittedly, a systematic study of the Poria carbonica hyphae distribution pattern in poles would be very desirable in order to establish more precisely the probability of missing an early infection. The evidence so far indicates a relatively rapid distribution of hyphae within the moist zone during the incipient stage of development.

In addition to its qualitative value, the culturing of field samples provides information on the type of fungi present: certain colonies sporulate quite obviously in contrast with the Basidiomycetes. At present three microbial population patterns have been found to prevail: 1) pure cultures of Poria carbonica, forming a dense white to gray homogeneous mycelial matt free from obvious spores; 2) pure cultures of F-y, the yellow pigment producing fungus with black conidia; 3) mixed populations, including, on occasions, bacteria together with the ubiquitous fungal colonies. These mixed cultures appear primarily near pockets of advanced decay.

At present only tentative conclusions may be drawn from these observations. The Poria carbonica infections call for rejection of the poles since no entirely satisfactory control treatment has been developed, though one may be available within a few months as

discussed in the control section. The same disposition applies to the poles infected with mixed microbial populations. The F-y infected poles may be held for further testing if possible; they may well become the longest lasting poles, as no damage to the tracheid wall results from this infection and it appears to prevent invasion by wood destroyers. The sterile poles may call for inoculation with F-y and/or spraying of any untreated wood exposed in the checks with a standard preservative solution.

Sampling should be repeated at intervals not exceeding two years because major deterioration of the heartwood is known to take place within that period of time (Hand, 1965) and no means of providing lasting residual protection against heartwood decay to poles in service has been yet demonstrated.

## CONCLUSION

The sampling-culturing procedure described detected successfully poles with early decay. Poles with advanced decay yielded a different fungal population than the poles at the incipient decay stage. More than fifty percent fungal infection was found in the poles sampled in service.

SECTION II - IDENTIFICATION:  
A - FLUORESCENT ANTIBODY TECHNIQUE

INTRODUCTION

The microbial population of wood appears to be relatively simple, although this concept may be influenced by the fairly selective culture media used for routine detection work, mainly malt extract agar at pH 5.5. Even when considering the limited microbial population revealed by this detection technique, a broad range of activities on wood constituents is found among the organisms isolated, calling for some sort of classification scheme. The usual approach to microbial classification is the matching of unknown isolates with the established species. In the case of fungi, species are defined almost exclusively in terms of morphological features; physiological properties are used only for supplemental characterization. Often the physiological properties considered are artificial and do not necessarily correspond to any significant feature in terms of natural microbionomics. As a result, recognized variations in the genotype of a species sometimes involve properties only accidentally related to natural microecological behavior, while critical changes may be overlooked. The traditional procedure for fungal classification is impaired further by the delays required for the complete observation of critical morphological features, which in some instances are never

available under practical working conditions, i. e. sporophores in Basidiomycetes.

Workers attempting to identify Basidiomycetes have frequently experienced a frustrating event: a given culture may show the typical clamp connections but no sporophore develops, preventing precise classification. A number of techniques have been proposed to induce sporophore formation among isolated Basidiomycetes, but none has been found consistently practical for routine identifications.

Furthermore the ecological significance of the present species concept is not clear. Pelczar (1958) expresses his views on the nature of current species thus:

. . . the arrangement of species into a system of classification, e. g. species → genus → tribe → family → order → class, might appear relatively easy and unequivocal. It is not. All the taxonomic categories for a particular group of organisms are based upon someone's judgment. The main units of biological classification, the species, has no objective definition. It is, in essence, a group of organisms so similar that most experienced microbiologists would agree they are alike. But the opinions of people differ; they may place different degrees of significance upon the characteristics of an organism; the species is man-made and exists only in the mind of man.

However, the traditional concept of classification is important because of its wide acceptance. For the purposes of this study a compromise was developed in that due concern was given to traditional speciation, but not to the traditional techniques used for classification. Instead the relatively new fluorescent antibody technique was explored.

This technique is based on the unique reaction of animals' internal environment to the invasion of large foreign molecules, particularly proteins. These invaders known as antigens trigger the formation of specifically conjugating molecules: the antibodies from the gamma globulin fraction of blood serum. Whenever antigen and antibody react a loss of solubility occurs allowing more effective disposal of the invading substance and its adjacent source, pathogenic bacteria for example, by the phagocytes of the infected animal.

The usefulness of the reaction, for identification purposes, is the high degree of specificity between antigen and antibody. Once a pure antibody has been obtained against a given antigen, it will conjugate only with an identical antigen. Thus, conjugation of a known antibody with any unidentified antigen or antigen bearing organism ascertains its nature. For identification purposes some tagging of the antibody is required in order to reveal its behavior upon contact with an unknown antigen. This is achieved with fluorescent dyes such as fluorescein isothiocyanate which have the dual ability of becoming attached to gamma globulins and also of glowing under ultra violet light (Bentner, 1961).

## REVIEW OF LITERATURE

The fluorescent antibody technique was first developed by Coons (1942) for the identification of pathogenic bacteria in situ. Many related techniques were developed for various bacterial species as described in a recent technique manual (Cherry, et al., 1960) but on only relatively few occasions were fungi identified in this fashion, particularly non-human pathogens. In human mycosis studies application of the fluorescent antibody technique is used for two purposes: 1) rapid diagnosis, 2) morphogenetic studies.

Kaufman and Kaplan (1963) studied the antigenic relationships between yeast and mycelial forms of Histoplasma capsulatum and Blastomyces dermatidis. Four antiglobulins were prepared which showed various degrees of affinity with the respective yeast and mycelial forms of these two pathogens. No distinct antigen was found specific for the mycelial cells of these two organisms.

Cerottini, et al. (1964) augmented this study by demonstrating the occurrence of three fractions in the gamma globulin fraction of anti Histoplasma capsulatum serum. Only two of these three fractions were shown to be specific against the fungus; the third fraction would not react regardless of the immunity of the rabbit.

Kaufman and Brandt (1964) developed fluorescent-antibody reagents to differentiate the mycelial form of Histoplasma capsulatum,

from several morphologically similar but saprophytic species of the genera Sepedonium and Chrysosporium.

Gonzalez-Ochoa and Kaplan (1964) reported the successful identification of Sporotrichum schenckii in pulmonary exudate smears in two hours with the fluorescent antibody technique instead of one week with the usual procedure.

Silva and Kaplan (1965) were able to differentiate between the yeast form and the mycelial form of Paracoccidioides brasiliensis with purified antiglobulins. The frequently troublesome cross staining was alleviated simply in this case by preadsorption of the non-specific antibodies with the conflicting organisms.

Fungal morphogenesis was studied with fluorescent antibodies by Gross and Summers (1964). The sites of cell wall extension as new cells develop in growing hyphae, and the origin of the daughter cell wall constituents, were demonstrated with this technique.

No published work on plant pathogens was found although Patton (1966) has had remarkable success in differentiating specific soil fungi from other soil microorganisms in culture, in root tissue, and in soil.

## MATERIALS AND METHODS

### Fluorescent Antibody Method

The indirect method was used in order to avoid the time consuming conjugation of antibodies with the fluorescent dye. Fluorescent rabbit antiserum (goat) was purchased from Microbiological Associates, Bethesda, Maryland, while antibodies against Poria carbonica were prepared in the facilities of the Microbiology Department with the guidance of Professor Pilcher and cooperation of David Henning.

### Culture of Fungal Antigens

Mycelial pellets of Poria carbonica #265, Poria xantha, Poria monticola, Fomes subroseus, Lenzites trabea, and Lentinus lepideus were grown in 250 cc shake flasks of malt extract broth for about two weeks. The medium contained 25 grams malt extract per liter of distilled water. All cultures were received originally from the U.S. Forest Service, Forest Products Laboratory, Madison, Wisconsin.

### Washing Antigen

After incubation, the mycelium was centrifuged at 3,500 rpm for ten minutes; the supernatant was poured off and replaced with

sterile physiological saline solution and the mycelium resuspended. This washing operation was repeated three times. The mycelium was then resuspended in 100 ml saline and ground for ten minutes at low speed in a Waring blender.

#### Killing Antigen Bearing Cells

The ground mycelium was centrifuged again and resuspended in 0.5 percent formalin solution in water. This solution was prepared from 100 percent formalin which contains 37 percent formaldehyde by weight.

#### Controls

The various fungal cultures used were plated on malt extract agar before use of the mycelial pellets to detect possible contamination. The effect of grinding and treatment with formalin was tested in the same fashion, Figure 8.

#### Packaging Antigen

After an overnight exposure at room temperature to the formaldehyde the ground mycelium was centrifuged once more and resuspended in a 1:20,000 phenyl mercuric nitrate in such proportion that a 0.4 percent by volume concentration of hyphal material, as measured with an hematocrit tube, was present in the suspension.



Figure 8. Grinding of the Poria carbonica mycelial pellets in a Waring blender retards their growth, but does not stop it; when formalin is introduced in the solution, the hyphae die within twenty-four hours.

The antigen preparation was then bottled in a glass vial, capped with the usual rubber hood, and kept in the refrigerator ready for use. Just before injection the antigen was diluted 1:1 with prepared Freund's adjuvant, bringing the hyphal concentration to about 0.2 percent by volume.

#### Adjuvant Preparation

The adjuvant acts as a synergist in the formation of antibodies in the inoculated animal. It is composed of mineral oil, wax and dead tubercle bacilli. It is usually prepared (Carpenter, 1965; Cushing and Campbell, 1957; Stafseth, Stockton and Newman, 1956) in a two step procedure: oil-in-water emulsion preparation and mixing with bacilli. The oil-in-water emulsion is prepared in double strength because it is to be diluted 1:1 with the commercial Freund adjuvant according to the following directions:

Sterilize good quality light paraffin oil and Arlacel A from the Atlas Powder Company, Wilmington, Delaware, by autoclaving. Mix 8.5 volumes of the paraffin oil with 1.5 volumes of Arlacel A, using a Waring blender or aspirating it repeatedly into a syringe without needle. Add 10 volumes of adjuvant and mix as before. Test the emulsion by allowing a drop to fall from an applicator stick onto the surface of water. If the drop remains perfectly formed and does not spread over the surface, the emulsion is ready for use.

### Inoculation of Animal for Antibodies

Two rabbits were injected subcutaneously, each with 1.5 ml of completed antigen suspension, in four different areas of the flanks, using a #21 needle. Each rabbit was immobilized in the usual restraining box during the injections. The second injection took place ten days later with 2 ml of antigen suspension at three different places on the back. One rabbit died within 48 hours after the second injection.

### Serum Preparation

The remaining rabbit was bled through the ear vein fourteen days later, withdrawing 50 ml of blood. The procedure was repeated after another ten days. Coagulation was induced by whipping of the blood. The serum was filtered through a Seitz apparatus, distributed in small vials in 0.5 ml quantities, and frozen until needed for staining.

### Staining

The indirect method was used, with the following procedure recommended by Microbiological Associates, the suppliers of fluorescent rabbit antiserum.

1. Fix antigen to slide: several methods were tried;

satisfactory results were obtained with the "squash" technique to spread the mycelial pellet over the glass slide, followed by heat fixation.

2. Unlabeled antiserum, obtained as described above, was applied for 30 minutes at 37° C, or 4° C overnight, in humid environment. High humidity was maintained by placing the slide in a petri dish with a water saturated strip of filter paper on each side.

3. Wash slides for ten minutes in buffered saline. Change saline three times.

4. Cover specimen with fluorescent labeled anti-rabbit globulin and place at 37° C for 30 minutes in humid environment.

5. Wash slides for ten minutes in buffered saline as in step three.

6. Remove excess saline and mount slides in buffered glycerol (90 percent glycerol buffered with pH 7.0-8.5). Place coverslip over specimen and examine under ultraviolet microscope.

7. For controls,

a. Replace unlabeled specific antiserum from rabbit with antiserum from different animal species. No staining should result.

b. Omit steps two and three and begin with step four. No staining should result.

c. Apply unlabeled normal rabbit serum to specimen, wash and apply labeled anti-rabbit globulin. No staining should result.

- d. Staining should be blocked or diminished by dilution of the conjugate in unlabeled anti-human globulin.

### Examination

The slides were examined under a Leitz microscope. The source of light was a mercury arc lamp, Osram XBO 200, in an Ortho Lux illuminator. Various filters were used at the light source and in the ocular to achieve partial visible light or ultraviolet light exclusively without harmful effect on the operator. The heat filter was left on during the entire examination period.

The filter arrangement was as follows: Heat filter "Streuscheibe" was on at all times and the condenser used was NA 1.20. For visible light, an orange brown filter was used in a Blau Abs (2.5 mm OGI) ocular; for partial ultraviolet light, the same arrangement was used as above with addition of a blue glass plate below the condenser; for complete ultraviolet light the ocular filter was changed to a UV Abs dunkel 2.5 mm Euphas, greenish yellow, while the light source received a US 2 mm UGI filter.

### Photomicrographs

An Exakta camera was used to take the black and white photomicrographs on Kodak Panatomic film. The exposure time ranged from 5 seconds to 120 seconds depending on the light intensity;

5 seconds with full visible light; 45 seconds with partial visible light;  
1, 1.5 and 2.0 minutes with ultraviolet light only.

## RESULTS

Figure 9 shows Poria carbonica after staining under partial ultraviolet light. Figure 10 shows Fomes subroseus under the same conditions and no glow appears. Four other species were examined in this fashion: Lentinus lepideus, Lenzites trabea, Poria monticola, Poria xantha. Only one, Lentinus lepideus, was nearly as intense in fluorescence as Poria carbonica. In addition to various fungal mycelia, wood particles were stained. Pronounced fluorescence was shown by the wood also.

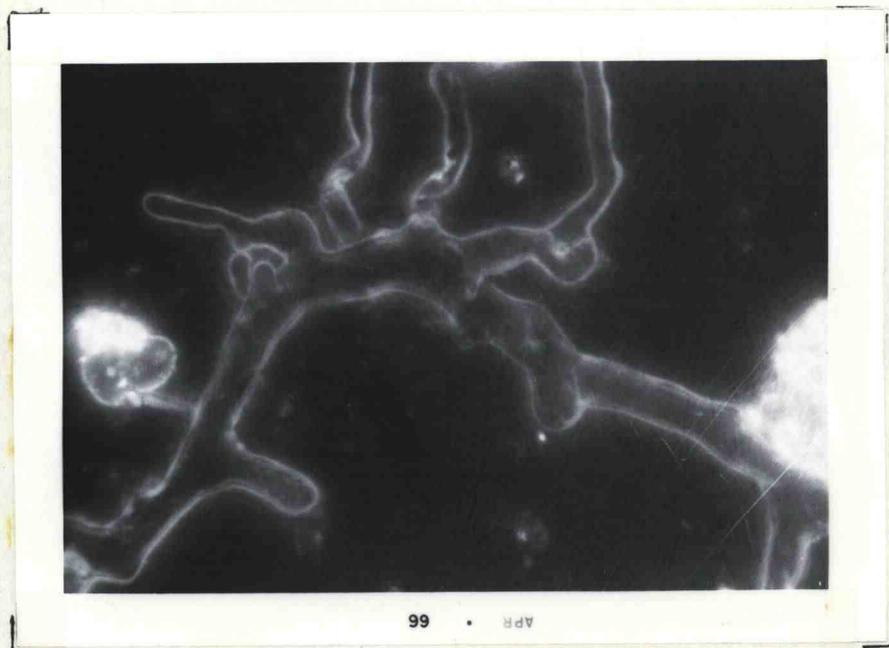


Figure 9. Poria carbonica glowing under partial ultraviolet light after staining with fluorescent antibodies, indirect method



Figure 10. Fomes subroseus under the same illumination as used in Figure 6 does not glow. This fungus did not retain the antibodies specific for P. carbonica.

## DISCUSSION

Specific fluorescence was obtained, indicating that *Poria carbonica* mycelium behaves as an antigen in the internal environment of rabbits. Cross staining occurred, limiting the effectiveness of the technique for present identification purposes. However, isolating two of the six alternatives tested has merit. No attempt was made to eliminate cross staining by such treatment as conjugation of the non-specific antibodies by pre-exposure to the non-significant fungi. This precaution was reported effective for Paracoccidioides by Silva and Kaplan (1965).

The behavior of wood under the conditions used seems to preclude the application of the technique to the identification of the fungi in situ. Culture on agar medium and subsequent preparation of slides will probably remain necessary unless the procedure is altered considerably. Dr. Patton (1966) suggested tagging with a fluorescent dye of sharply contrasting wavelength from that of the wood, rhodamine, for example.

## CONCLUSION

Provided cross staining is successfully controlled, the fluorescent antibody technique appears promising for identification of fungi, especially those which do not form sporophores readily under artificial conditions. When properly applied, the technique may reduce the identification time from weeks to a few hours and in some cases may allow a degree of specificity beyond the reach of standard classification techniques.

Even without further refinement, the fluorescent antibody technique greatly simplified the identification of Poria carbonica mycelium.

SECTION II - IDENTIFICATION:  
B - TRACHEID WALL LYSIS

INTRODUCTION

A more pragmatic approach to identification was studied at the suggestion of Dr. T. C. Scheffer, U. S. Forest Products Laboratory, Madison, Wisconsin. The primary concern about fungal incidence in poles involves their effect on the mechanical strength of the pole, which is due--at the cellular level--to the components of the tracheid wall. Deterioration of these walls is the reflection of complex biochemical reactions involving chiefly the interaction between fungal exoenzymes and the lignin-cellulose constituents of the tracheid wall. Through hydrolysis these large molecules are separated into their "building blocks," which may then be taken up across the hyphal wall and utilized for exergonic reactions or merely transported away in solution from the point of impact of the hyphal tip on the tracheid wall. This lysis does not take place in the same fashion with all wood inhabiting microorganisms. The "trade" classification of wood inhabiting fungi into white rot, brown rot, soft rot, and stain fungi is, in large part, an acknowledgment of that fact on the basis of observations at the systemic level. At the cellular level it is quite obvious and the term "bore hole" was coined to describe the effect of certain fungal hyphae on wood cell walls. Techniques for evaluation of this

effect at the molecular level are available but at this point seemingly not as convenient as the thin section technique for observation on the tracheid wall. Cell examination of the increment borer core cultured for the development of fungi, if present, provides a convenient substrate for the evaluation of the tracheid wall lysis by the permeating hyphae.

## REVIEW OF LITERATURE

As early as 1833, T. Hartig described in detail hyphae passing through the walls of wood cells. Over the years Unger, Willkoman (1866) and especially R. Hartig (1894) expanded on this work. The enzymes involved were studied by Czapek (1899), establishing the chemical nature of wood decay. Zeller (1916) listed a number of enzymes found in the mycelial mats of Lenzites saepiaria; these included cellulase, ligninase and hemicellulase.

Proctor (1941) made extensive studies of hyphal penetration through tracheid walls, describing the openings found as a result of chemical action of the fungal hypha as bore holes. His main source of data were ultraviolet light photomicrographs at various magnifications. The major aim of his study was the demonstration of biochemical lysis of wood cell walls by xylophagous fungi, as opposed to the mechanical action suggested by various workers. Six species of fungi were used in his investigation: Fomes annosus, Fomes pini, Lenzites trabea, Polyporus schweinitzii, Poria weirii and Trametes serialis. Four wood species were inoculated with these organisms: eastern white pine, Pinus strobus; western hemlock, Tsuga heterophylla; Douglas-fir, Pseudotsuga taxifolia; western red cedar, Thuja plicata. He concluded that

. . . penetration of the walls of wood cells by the hyphae of wood destroying fungi is accomplished by (1) the secretion

of enzymes at the tips of penetrating hyphae and (2) the total local dissolution of the cell wall by enzymatic activity in advance of actual passage through the cell wall. . . . In all cases of penetration observed, the tip of the hypha was preceded by a cavity of significant proportions. . . . Careful examination of hundreds of bore holes, some with polarized light, failed to disclose evidence of any kind that mechanical force is an instrumentality in the penetration of cell walls of wood.

These observations were augmented considerably with electron microscopy by Liese and Schmid (1962) "on brown-rot, white-rot, white-pocket rot, soft-rot, fungi and blue-stain fungi." These authors found that the decomposition of the lignin moiety of the cell wall varies with the different types of decay fungi, though the cellulose hydrolysis is similar in all types.

Lignin decomposition is the prerogative of white rot fungi, together with the ability to lyse all other tracheid wall components, resulting in a complete breakdown of the wood cell wall.

Cellulose hydrolysis is indicated by the appearance of rhombic or rhomboid structures. This is characteristic of the soft-rot, although it occurs also with the brown and white rot fungi in a less obvious fashion.

Blue stain fungi exude no enzymes inducing wood cell wall lysis. Their hyphae penetrate the membrane of bordered pits or tori without constriction. When crossing a tracheid wall a specialized bore-hypha develops from an appressorium, formed by the bulging of the usual hypha.

The outstanding conclusion from Liese's work is perhaps the explanation of the wood wall penetration mechanism by blue stainfungi. This was demonstrated further in another publication by the same authors (1964) with hyphal penetration in silver and aluminum foils. The term "transpressorium" was proposed to define the penetrating hypha and includes a stalk, a head and a peak.

Blue stain fungi penetration across tracheid wall, bordered pit and also wood ray cell was discussed further (1961) in a separate publication. The previous reports that bore holes from these organisms showed no evidence of tracheid cell wall dissolving enzymes were confirmed.

Waterman and Hansbrough (1957) made an extensive study of brown-rot and white-rot fungi in an attempt to determine whether reduction in toughness caused by wood-decay fungi could be estimated from the changes observed microscopically in the tracheid wall. The relation was rather variable, though there was an association of average values for both factors. The brown-rot fungi studied were: Poria monticola, Polyporus schweinitzii, Polyporus sulphureus, Lentinus kauffmanii; for white-rot decay studies the following organisms were selected: Fomes pini, Poria subacida, Polyporus borealis. The brown-rot deterioration induced by this fungus is described as follows:

The hyphae of rapidly growing isolates, such as those of P. monticola, formed conspicuous bore holes during the first two weeks of culture. The hyphae of other species usually passed from one tracheid to another through the bordered pits during the early weeks of culture, but later produced bore holes.

In the early stages of decay caused by the white-rot fungi, the hyphae usually passed from one tracheid to another through the bordered pits, without visible effect on the walls of the pits or on the tracheid walls.

After eight weeks in culture, the progressive decomposition of the cell walls of tracheids and medullary ray cells was indicated by a gradual thinning of the walls, and particularly by the reaction of the Pianeze IIIb stain.

Soft-rot fungi were studied by various workers, particularly Duncan (1965), who described their effect on the cell wall as follows:

. . . the hyphae ramify within the cell wall, and make tunnels that run longitudinally and follow the cellulose fibrils. . . . In cross sections, the cavities appear as holes that equal or exceed the diameter of the hyphae.

Staining fungi have also received much attention. Scheffer (1940) reports that

. . . the strong tendency of sap stain fungi to follow the rays leads to a very characteristic distribution of stain in wood, which is particularly noticeable in materials of comparatively large size. . . . The discolored areas on the ends are almost invariably wedge-shaped with the apex of the wedge pointing toward the pith.

Since the rays are not continuous, stain fungi complete their penetration by growing to some extent into and through the fibers or tracheids, as the case may be. This is accomplished by passage of the hyphae through the pits in the cell walls or, less frequently, by direct penetration of the walls. . . . In most cases the part of the hyphae that traverses the wall is considerably smaller than the remaining portion in the cell cavity. Hubert (1929) has suggested that the undersized portion of the fungus within the wall represents the original diameter of very young hyphae, which are responsible

for the penetration, enzymes at the hyphal tips dissolving the wall at the point of contact.

Lagerberg, Lundberg, and Melin (1928), however, feel that the evidence points toward direct penetration by native hyphae, which are simply constricted in passing through the wall. Their discussion of this subject also suggests the possibility that penetration may be accomplished mechanically through the aid of appressoria. In any event, there is little evidence of extensive enzymatic action, such as is reflected in the large bore holes made by some of the wood-rotting fungi.

Evaluation of comparative exoenzyme activity is fairly accurate through microscopic examination, but the complete absence of enzymatic secretion is rather difficult to establish in this way. Chemical analysis of the wood constituents at various levels of infection may prove more convincing.

A somewhat similar pattern in tracheid wall lysis is described by Lindgren (1952). When Trichoderma viride attacks southern pine, "a partial or complete breakdown of the ray parenchyma cells takes place, but the tracheid wall appears untouched."

Table IV summarizes the major features described by the various workers mentioned. The effect on wood strength of the brown- and white-rot is not directly proportional to their "bore hole" activities as established by Waterman and Hansbrough (1957), but, according to Cowling (1961), rather to the microscopical action of the cellulase. Cowling (1961) states that

. . . although experimental proof is lacking specifically for wood, physicists have demonstrated that the strength of other cellulosic materials may be attributed largely to cellulose

with a high degree of polymerization (Hermans, 1949). Any treatment that drastically decreases the length of cellulose molecules may be expected to decrease the strength properties of the material correspondingly. The rapid and very gradual changes, respectively, in average degree of polymerization of the cellulose in brown- and white-rotted wood correspond with and provide an explanation for the generalization that, at comparable losses in weight due to decay, the strength properties of brown-rotted wood are much lower than those for white-rotted material.

The brown-rot fungus, Poria monticola, primarily utilized the carbohydrates . . . ; lignin was metabolized only to a minor extent. The rate of utilization of each sugar polymer present in the wood remained essentially constant in all stages of decay, and it was approximately proportional to the amount present in sound wood. The alpha-, beta-, and gamma-cellulose fractions of the holocellulose were utilized, each at a different rate in all stages of decay. Each of these conclusions is supported by direct measurement of the amount of each constituent present in the wood in progressive stages of decay by P. monticola.

In the initial stages of decay, by P. monticola, the wood constituents were depolymerized much more rapidly than the degradation products were converted to volatile products of respiration. This conclusion, as applied to the wood carbohydrates, is supported by the following evidence: (1) the extreme rapidity of decrease in average degree of polymerization of the holocellulose, 78 percent decrease in average 'degree of polymerization' after only 10 percent weight loss; (2) the rapid decrease in alpha-cellulose and accompanying increase in beta-cellulose content of the wood; (3) the rapid accumulation in the wood of materials extractable in all the solvents used in the solubility analyses. The validity of this conclusion as applied to lignin is demonstrated by the very slight loss of lignin from the wood and the accompanying increase in the amount of apparent and sulfuric acid lignin in the water and 1 percent alkali extracts.

The white-rot fungus, Polyporus versicolor, utilized each of the major constituents of sweetgum sapwood in all stages of decay. The rate of utilization of each constituent was essentially constant in all stages of decay, and approximately proportional to the amount present in sound wood. These conclusions are demonstrated by direct measurement of the

amount of each constituent present in progressive stages of decay by P. versicolor.

In all stages of decay by P. versicolor, the wood constituents were depolymerized only about as rapidly as the depolymerization products were converted to volatile products of respiration. . . . The residue that remained after various stages of decay by P. versicolor had substantially the same composition and many physical properties in common with sound wood.

Table 4. Main wood inhabiting fungi.

Visible effect on wood <sup>1</sup>	Common name	Typical species	Class	Preferred habitat		Effect on tracheid wall <sup>2</sup>			Effect on wood strength* <sup>3</sup>
				Moisture	Type wood	Penetration pattern	Lysis around hyphae	Appearance at 100X	
Deterioration	Brown rot	<u>Poria carbonica</u>	Basidiomycetes	High, i. e., > 20% < Saturation	Heart-wood	Transversal	Extensive, up to 2 - 3X $\phi$ hypha	Bore holes	high and greater than expected from visible decay  high, but proportional to visible decay
	White rot	<u>Polyporus versicolor</u>			Sap-wood				
Softening	Soft rot	<u>Chaetomium globosum</u>	Ascomycetes, also fungi imperfecti	Very high, i. e., saturation	Sap-wood	Longitudinal	Medium 1.0 - 1.5X $\phi$ hypha	Holes in middle lamella cross section	high, proportional to visible decay after hard surface or "shell" is removed
Staining	Fungal stain	<u>Ceratomyella pilifera</u> , <u>Endoconidophora coerulescens</u>	Fungi imperfecti	High > 20% < saturation	Sap-wood	Usually through bordered pits, occasionally across tracheid	None, penetrating hypha shows $\phi$ inside < $\phi$ outside wall	Hyphae "squeeze" through wall	Little if any
None	F-y	Unidentified	Fungi imperfecti	Low, i. e. < 20%	Heart-wood				

In the text, these events are described as: <sup>1</sup> systemic level, <sup>2</sup> cellular level, <sup>3</sup> molecular level

\*This is a reflection of the visible deterioration at the systemic and cellular levels, and also of the amount of cellulase secreted by the penetrating hyphae; though not an accurate means of measuring loss of wood strength. Cowling indicates that it can be measured more accurately by the DP - degree of cellulose polymerization - in the infected wood than by visual examination even under magnification.

## MATERIALS AND METHODS

Work in this area was done with the cooperation of Dr. R. L. Krahmer of the Forest Research Laboratory. Thin sections were prepared from pole cores cultured for two weeks on malt extract agar for incipient decay detection or wood sticks placed for three weeks on the surface of a malt extract agar plate inoculated with fungi. Razor blade and sliding microtome were both used for the preparation of the thin sections. The optimum thickness appears to be about 30 microns.

The Pianeze IIIb stain described by Wilcox (1964) was used in most cases. The Picronaniline procedure was tried but did not stain the smaller, presumably monokaryotic, hyphae of the fungus.

A modification of the Bismarck brown stain described by Hubert (1922) was used with the F-y organism because the other procedures failed to provide sufficient contrast between wood and hyphae for detailed observations and photomicrographs. The following procedure was selected after a number of trials using various exposure times, sequences and concentrations:

1. Soak the wood section in methyl violet two hours.
2. Wash successively in 15, 30, 50, 70 percent by volume ethanol solutions.
3. Soak in 95 percent by volume ethanol solution containing the Bismarck brown dye.
4. Wash in colorless 95 percent ethanol, then 100 percent ethanol.

5. Use xylene prior to the final step of mounting in HSM medium.

The wood was stained brown and the hyphae purple.

## RESULTS

Poria carbonica apparently attacked all the components of the cell wall, leaving conspicuous "bore holes" when the tracheid is examined longitudinally (Figure 11).

Some bore holes no longer appear to contain hyphae providing an explanation for the lack of live fungi recovery from advanced decay zones.

The diameter of bore holes appreciably exceeds that of the hyphae.

Cross sections of infected tracheids show extensive damage by enzymatic action. Certain sections showed haustoria-like structures (Figure 12) developed within the ray cells by the infecting fungus.

F-y showed an entirely different activity on the tracheid wall; no bore holes could be found in wood sections showing extensive hyphal development and sporulation in cell lumens (Figure 13). Cross sections did not reveal any change in the tracheid wall as a result of F-y growth (Figure 30). The penetration of hyphae through walls was infrequent, and whenever occurring did not cause any peripheral lysis (Figure 14). The hyphal segment actually embedded within the wall was definitely smaller in diameter than the hyphae present in the cell lumen (Figure 15). Immediately before and after contact with the tracheid, the hyphae appeared to develop an accumulation of cytoplasm in the shape of a suction cup.



Figure 11. Bore holes show as white dots in this thin section, where a *Poria carbonica* hypha crossed the tracheid wall. These bore holes reveal the wood destroying properties of the fungus.

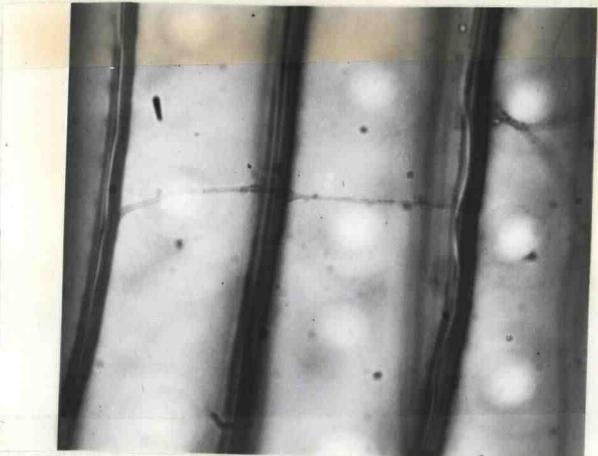
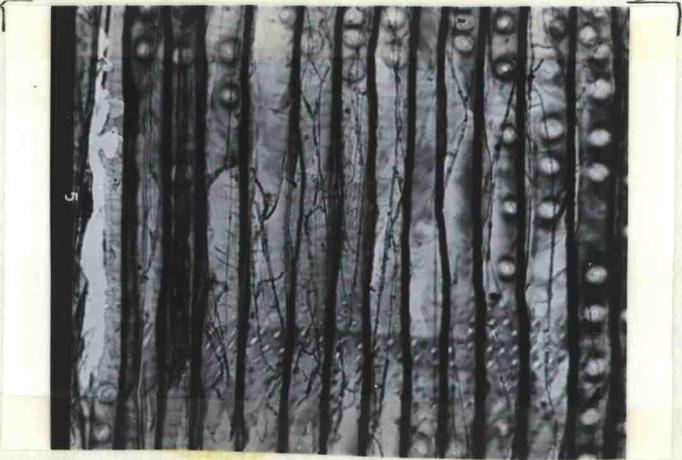


Figure 12. Two types of *P. carbonica* hyphae were seen in certain sections. The larger hyphae showed well after counter staining with picroaniline blue, while the smaller hyphae were visible only when safranin was used exclusively.

Figure 13. These F-y hyphae permeated the tracheid over an incubation period of three weeks. The mycelium is visible primarily in the cell lumina, little tracheid wall crossing occurs.

Figure 14. The occasional penetration of an F-y hypha through a wood cell wall shows no enzymatic lysis.

Figure 15. Penetration through a tracheid cell wall induces distinct morphological changes in the F-y hypha, but no damaging lysis on the wood.



## DISCUSSION

The effect of the Poria carbonica hyphae on the tracheid wall is obviously quite different from that of the F-y and appears to provide one of the "expedient boundaries" called for in taxonomy. The broad differences in fungal behavior when penetrating tracheid walls have been reported for many years and studied in increasing details as more refined instruments, particularly the electron microscope, became available. Tracheid wall lysis thus provides a reliable criterion to define fungal behavior.

Table IV lists the major type of wood inhabiting fungi and includes their tracheid penetration pattern. Poria carbonica in this respect also is typical of brown rot fungi while F-y resembles stain fungi in its tracheid penetration. It differs from staining fungi in other respects, however, particularly in habitat: F-y is found in the heartwood as well as in the sapwood of Douglas-fir poles.

It has been shown by Waterman and Hansbrough (1957) that bore holes and loss of mechanical strength are not correlated directly. Cowling (1961) explained the much greater impact of brown rot fungi on wood strength by the random depolymerization effect of their exocellular cellulase.

Even in the most advanced stage of decay, the average DP of the white rot holocellulose was 1,360 DP units, or 83 percent of the sound wood value of 1,635. This effect is expressed in DP units based upon the degree of polymerization as calculated

from intrinsic viscosity measurement. This same stage of depolymerization occurred in brown-rot at an estimated weight loss of only 1.5 percent. The average DP of the brown-rot holocellulose reached 70 DP units, or 4 percent of the sound wood value, at 70 percent weight loss.

Bore holes then do not indicate just how serious a threat the organism presents; in all cases, however, it indicates a wood destroyer. On the other hand, staining fungi have no lytic effect on the wall and have no effect on the mechanical strength of the wood. The tracheid wall lysis offers a convenient criterion for evaluating wood rotting potential for fungi developing from the cores cultured for the detection test. Upon incubation, the infected cores can supply thin sections for the examination of tracheid wall lysis induced by the organism present. Both longitudinal and cross sections should be used to detect soft-rot as well as brown- and white-rot organisms.

## CONCLUSION

Tracheid wall lysis provides a convenient means for evaluating the wood destruction potential of fungi developing from cores cultured for the detection of incipient decay.

Poria carbonica produces conspicuous bore holes similar to those reported for brown- and white-rot fungi, while other fungus species do not. The fungus F-y, for example, appears to rely on mechanical force to penetrate the tracheid wall while moving from one lumen to the next.

## FOREWORD TO SECTION III

Two appreciably different approaches to the control of Poria carbonica were studied; chemical control with gases and biological control, first with bacteria, then with an antagonistic fungus. Although these two approaches may eventually complement each other in the field, they are presented here separately.

## SECTION III - CONTROL: A - CHEMICAL CONTROL

## INTRODUCTION

Chemical control is needed for those poles in which decay fungi have been detected in the heartwood but which have not deteriorated to the point of danger so far as mechanical strength is concerned.

Ideally the control technique should be suitable for the treating of poles in service, destroying the fungi present, and providing sufficient residual action to prevent reinfection. It should also be economical and safe. To be suitable for use in human communities, it should compare well with known pesticides to deserve exemption from discriminatory regulations. Although the chemical control study was concerned particularly with "in service" treatment, it need not necessarily be limited to this situation. It might also be used to treat poles prior to the conventional pressure treatment process, and, conceivably, as a new process for treatment of new wood products.

## REVIEW OF LITERATURE

A number of techniques have been developed for the treatment of poles in service, especially in the ground line zone where the combination of moisture and oxygen make fungal growth possible (U.S. Forest Service Wood Handbook, 1955). Most of these methods affect only the fungi present at the surface of the pole and therefore have little value for the control of heartwood decay. The most widely used is perhaps the charring and creosote spraying method with more than 100,000 poles treated in that fashion.

Although no gas application appears to have been reported for the treatment of poles, fumigants have been studied for the destruction of forest pathogens in order to meet import requirements established by several countries. Partridge (1961) tested 15 fumigants for ability to kill Ceratocystis fagacearum and to penetrate small sections of black and white oak wood. A third test involved penetration in larger sections three and four inches in diameter and eight inches long with intact bark. These applications were made under atmospheric pressure and at room temperature. Penetration was measured by the killing of the fungus within the oak sections treated. Only chloropicrin and methyl bromide were effective when three-day exposure periods were used. Jones (1963) reported application of methyl bromide in the field to larger logs 12 to 17 inches in diameter.

The treatment lasted seven days at atmospheric pressure and temperatures ranging from 65 to 91° F in one instance, 38 to 90° F in the other. No live fungus was recovered from any of the fumigated logs, while 41 percent of the samples from one set of controls and 64 percent from another set showed live oak wilt fungus.

Quite recently, gas treatment (Liu and McMillin, 1965) has been applied to wood with some thoughts of "increasing the resistance for deterioration of wood and its derivative material," though the primary consideration is "minimizing changes in physical dimensions and deterioration of mechanical properties resulting from variations in the moisture content of materials of this kind," namely, wood. In all examples cited, as well as in the general description, the process calls for evacuation of air from the wood through exposure to a vacuum of 29 mm of mercury absolute in an autoclave or similar hermetic vessel. This is essential presumably for the penetration to some depth of the relatively bulky ethylene oxide molecules within the tracheids and for actual collision with the hydroxyl groups of the cellulose molecules.

## MATERIALS AND METHODS

The materials used for the chemical control experiments were gases: methyl bromide ( $\text{CH}_3\text{Br}$ ) and ammonia ( $\text{NH}_3$ ). The methods varied with the purpose of the four types of experiments performed: (1) effect of methyl bromide on Poria carbonica, (2) penetration of methyl bromide within poles, (3) field test of methyl bromide on pole in service, (4) field test of multi gas diffusion (ammonia, oxygen, and methyl bromide) on used poles out of service.

### Effect of Methyl Bromide on Poria carbonica

The effect of methyl bromide on Poria carbonica was determined under laboratory conditions by exposing infected wood cores and malt extract agar cultures of Poria carbonica to a measured weight of 100 percent methyl bromide. Nine infected wood cores three-eighths by six to eight inches were broken into 20 pieces of equal size and divided evenly between two sets of agar plates: one for treatment with methyl bromide, the other for direct incubation. The samples were placed in a cylindrical pressure-cooker of 0.91 cubic foot capacity and nine grams of methyl bromide were introduced from a "lecture-type" pressure bottle (net weight 1.5 lb) through a section of tygon tubing. A torsion balance was used to measure the gas released. The relative humidity was maintained near saturation by evaporation

of distilled water from a beaker. The treatment lasted six hours under atmospheric pressure at a temperature of 25 ° C. No evacuation of air was attempted.

#### Penetration of Methyl Bromide Within Poles

Nine used pole sections ranging from 8 to 19 inches in diameter and 2' 4" to 5' 0" in length were placed in a fumigation vault consisting of a gas tight concrete chamber equipped with ventilation equipment, gas valves, and fumigant heater. The ends of the pole sections were coated with hot liquid paraffin to prevent longitudinal penetration of the gas. Two holes approximately 1/4 inch in diameter were drilled in each section in the same vertical alignment at about 1/4 the overall length of the pole from each end (Figure 16). A third hole was drilled at midpoint 48 hours after the gas release for further testing of gas penetration. These facilities were made available by the Ardee Pest Control Service in Portland, Oregon, on their premises.

Sections of tygon tubing were introduced into the holes, pushed to the bottom, and sealed with masking tape and liquid paraffin at their point of entry into the poles. The sections of tubing were attached to a gas measuring device, a "Fumoscope," distributed by Neil MacLean Company. Methyl bromide was released into the vault at the rate of 10 lbs. per 1000 cubic feet. Fumoscope readings were taken at various time intervals during the 24 hours exposure period.

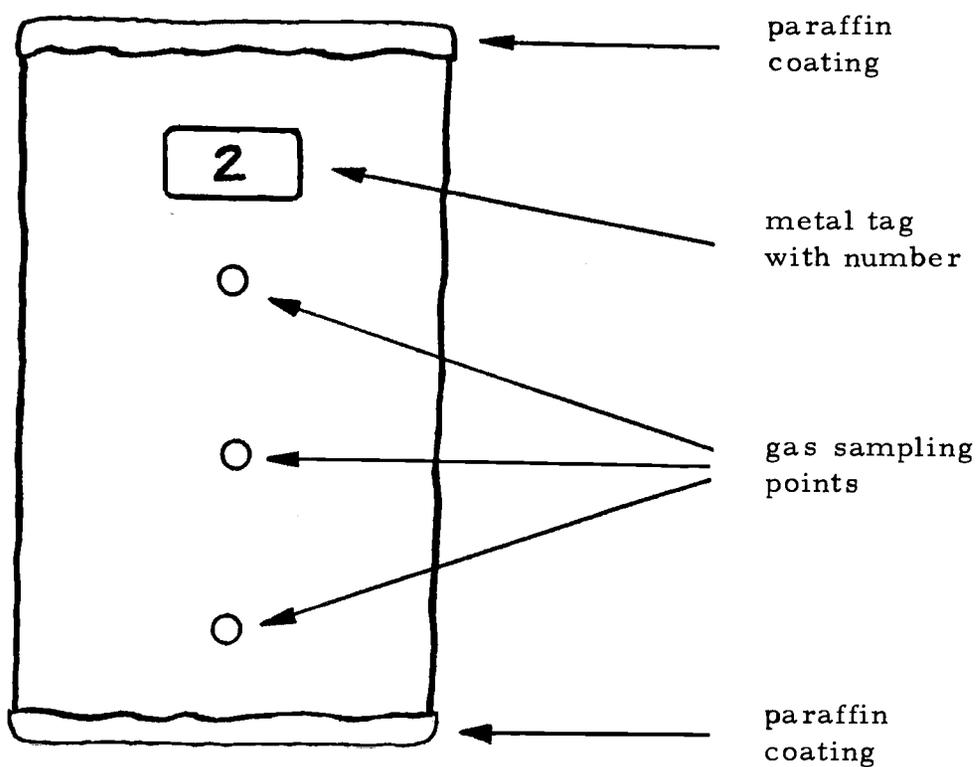


Figure 16. Pole section prepared for the penetration experiment with methyl bromide: the 2 end sampling points were drilled before exposure to the gas, tygon tubing was inserted and sealed in these holes for recording of the  $\text{CH}_3\text{Br}$  concentration during treatment. The center hole was drilled 48 hours after the end of exposure as a control for the readings obtained on the other two sampling points.

Upon completion of the fumigation, the vault was ventilated and readings taken on the methyl bromide concentration at the center of the poles 48 hours later. The temperature ranged from 54 to 78°F within the vault during the exposure period.

#### Field Test of Methyl Bromide on Pole In Service

A pole, No. 14E16, known to be heavily infected with Poria carbonica in the incipient stage was located in a convenient location on University property. The ground around the pole was removed to a depth of 18 inches and a width of one foot. Three moisture readings were taken with the Delmhorst Instrument Company's Moisture Detector Model RC-1, and three cores were removed for oven dry weight determination.

Six 2 x 4 in. boards four feet long and tapered on the last foot of each end were nailed on the narrow edge in a vertical position to the pole at regular circumferential intervals so as to form a "rib cage" around the ground line zone of the pole, i. e. starting at about one foot below the ground level and reaching up about three feet above. The "ribs" were then covered with six mil polyethylene sheet sealed at all joints with masking tape, adding sealing compound where cracks or seasoning checks occurred (Figure 17).

The lower end of the plastic sheet was sealed against the pole with soil packed mechanically and soaked with water for further

compaction. A hole was then punctured in the plastic "tent" around the pole for the introduction of a plastic tube linking the inside of the tent with a 1-lb. can of technical grade methyl bromide equipped with the usual puncturing device and valve assembly (Figure 18). A total of 2 lbs. of  $\text{CH}_3\text{Br}$  was used for a 24 hour period. The temperature ranged from  $68^\circ\text{F}$  ( $= 20^\circ\text{C}$ ) at the start to less than  $50^\circ\text{F}$  ( $= 10^\circ\text{C}$ ). The diagram (Figure 15) shows the sampling pattern of the nine cores taken out to assess the condition of the fungi before and after fumigation.

#### Field Test of Multi-Gas Diffusion on Used Poles Out of Service

Six poles were selected among a large number of used poles previously found to be infected with decay fungi. These poles were heavily infected with live Poria carbonica. They were laid in a metal tank normally used for dip treatment. A forced draft electric heater was installed at one end in order to maintain the temperature within the treatment area above the boiling point of methyl bromide  $38^\circ\text{F}$  ( $= 3.5^\circ\text{C}$ ). Poles and heater were covered with a six mil polyethylene sheet. The contact area between metal and plastic was made gas tight by the weight of sand poured on the sheet alongside the pile of poles. Figure 18 shows the overall arrangement just described as well as the position of the plastic tube linking the enclosed area to a

75-lbs. liquid ammonia pressure tank. Methyl bromide was released from 1-lb. cans through the same tube. The ammonia vaporized (B.P. - 33.4°C) slowly without difficulty under the ambient temperature but methyl bromide did not. This problem was overcome by plunging the can attached to the tube into a bucket of warm water. In addition to speeding vaporization this arrangement has the advantage of showing whether any gas leak is taking place around the can puncturing device and tube connection. The amount of gas released was measured by weight loss in the case of ammonia and 1-lb. can increments for methyl bromide. A total of 8 lbs. of ammonia and 6 lbs. of methyl bromide was released, starting with 2 lbs. ammonia, followed immediately with 4 lbs. of methyl bromide and concluded with 3 lbs. of ammonia in the evening, then repeating the following morning with a 2-lb.  $\text{NH}_3$ : 2-lbs.  $\text{CH}_3\text{Br}$ : 1-lb.  $\text{NH}_3$  sequence to compensate for leakage during the night, before sand was relied upon exclusively for the plastic-to-metal seal. At first, masking tape was used on two sides. It did not adhere well enough to the cold metal tank for the purpose. The last pound of  $\text{NH}_3$  was used for leak detection purposes (white vapor and odor) rather than for effect on wood. The overall exposure lasted 60 hours. The temperature within the "tent" remained appreciably higher than that of the surrounding atmosphere due to continuous operation of the electric heater. The inside tent temperature was recorded at 47°C at the end of the gas exposure

period while the atmospheric temperature was 24°C. Ammonia was still present in the enclosed area at the end of the exposure time.

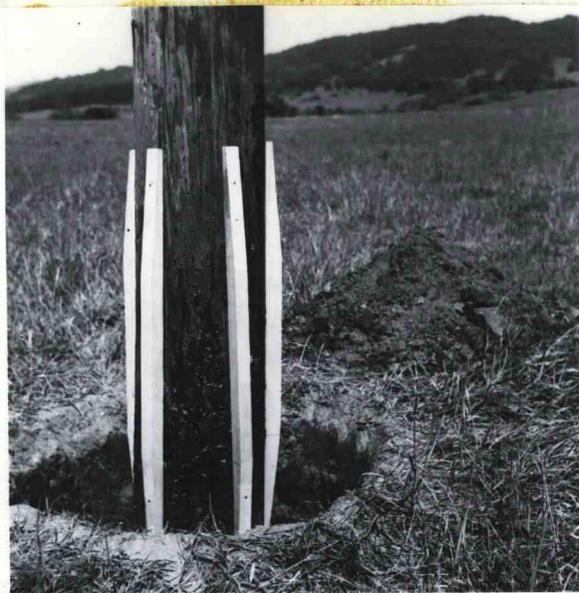


Figure 17. In service treatment of *P. carbonica* infected pole: the ground is excavated and the plastic sheet supporting wood frame is nailed in place.



Figure 18. In service treatment of *P. carbonica* infected pole: the plastic sheet is taped in place and the can of methyl bromide is linked to the plastic enclosure with plastic tubing. Gas treatment is ready to proceed.

## RESULTS

Effect of Methyl Bromide on *Poria carbonica*

Figure 19 shows representative samples of the twelve plates inoculated simultaneously: six with *P. carbonica* strain No. 89\*, six with strain No. 90\*. One-half of each set was treated with methyl bromide, while the other half was incubated without treatment. In every case the exposure to methyl bromide stopped growth of the fungus.

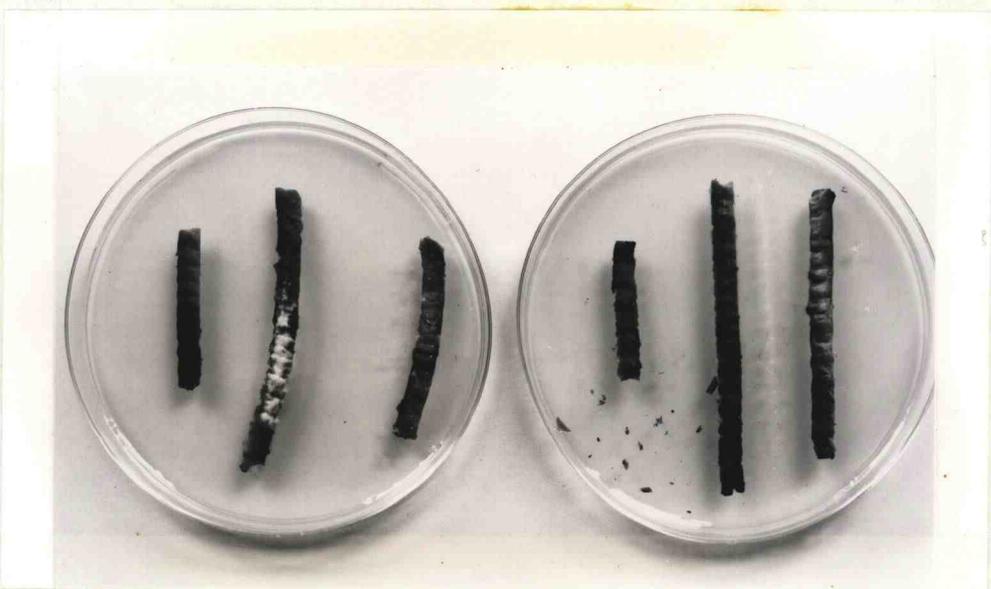
The methyl bromide treatment was repeated using wood cores freshly removed from an infected pole, No. 14E16, and similar results were obtained: no fungus growth resulted with the samples exposed to  $\text{CH}_3\text{Br}$  while the untreated samples showed profuse growth (Figure 20). The distribution pattern of the cores in the various plates precluded a chance event in the growth pattern of the fungus from the various core sections. The possibility of an interaction between methyl bromide and the malt extract agar used to grow the fungus was evaluated by the inoculation of plates just exposed to methyl bromide: growth took place as readily as in untreated medium.

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\* Bonneville Power Administration, Materials Testing Laboratory,  
Code No.

Figure 19. Methyl bromide is lethal for an agar culture P. carbonica strain #91 upon six hour exposure in moist atmosphere at relatively high concentration, i. e. more than ten pounds per 1000 cu. ft.: A is the treated culture, B is the untreated control.

Figure 20. Wood does not provide protection to P. carbonica hyphae against methyl bromide. These cores were infected equally; the group treated with gas no longer yields growth in culture.



### Penetration of Methyl Bromide Within Poles

The data of Table V show that in 30 minutes or less the methyl bromide had penetrated radially to such an extent that equilibrium was reached with the surrounding atmosphere in five pole sections. The other pole sections, the four larger ones (18 and 19 inches in diameter), had already absorbed a minimum of 26 oz. After six hours, the smaller poles still showed more than 100 oz. of methyl bromide while the surrounding concentrations had begun to drop, indicating leakage in the presumed gas-tight vault; the larger poles had increased their gas absorption to a minimum of 45 oz. and a maximum of 61, approaching the surroundings in gas concentration. In 24 hours of exposure the gas concentration in all poles had started to decrease following the change in the surroundings. The range between larger and smaller poles had narrowed down to 14 oz. differential. In a few poles the gas concentration appeared lower than in the surrounding atmosphere. This was probably due to the relative position of the sampling point with respect to the leaks in the chamber as well as the tendency of the methyl bromide to settle in the lower portion of the space available.

The results obtained might have been erroneous because of leakage at the sampling points around the masking tape seal of the tubing penetrating inside the pole section. Another set of samples was

Table V. Penetration of methyl bromide within poles  
Pole Sections Characteristics

		Number									Control (Space in Vault)	
Dimensions		1	2	3	4	5	6	7	8	9		
Methyl Bromide Concentration*	In Inches											
	Diameter	18	13	11	8	11	10	19	18	19	--	
	In Inches											
	Length	57	55	35	60	49	45	28	34	34	--	
	30 min.	Top	40	100+	100+	100+	100+	100+	35	27	34	
		Bottom	26	100+	100+	100+	100+	100+	29	29	38	100+
	6 hrs.	Top	55	100+	100+	100+	100+	100+	56	58	54	
		Bottom	61	100+	100+	100+	100+	100+	59	45	59	69
	24 hrs.	Top	44	44	46	44	50	46	41	46	41	
		Bottom	40	44	45	44	51	47	43	53	39	46
	48 hrs. after aeration of vault gas concentration at center pole, i. e. at bottom of 3rd hole (midpoint) 9 to 15 oz.											

\*Expressed in ounces per 1000 cu.ft., dosage applied 10 lbs. per 1000 cu. ft. The maximum concentration registered by the "Fumoscope" is 100 oz./1000 cu. ft.

obtained to evaluate such a possibility: a third hole was drilled well after the methyl bromide concentration in the vault was down to less than 1 oz. per 1000 cu. ft. When this third hole was drilled at mid-point in each of the pole sections, methyl bromide was found in every case ranging from a minimum of 9 oz. to a maximum of 15 oz.

This experiment indicated that the well-known penetrating ability of methyl bromide applied to Douglas-fir as well as to the other tightly packed commodities to which this gas is frequently applied.

#### Field Test of Methyl Bromide on Pole In Service

Pole 14E16 was selected for the field trial with methyl bromide because of its convenient location, in a fenced pasture, near the campus, away from buildings and safe from surprise encounters by pedestrians.

The moisture meter readings showed more than 25 percent moisture as is usually the case in decayed heartwood. One of the three samples taken for oven dry weight determination showed visible decay. All the other samples were for determining the occurrence and viability of the fungal population. Figure 21 shows the distribution on the pole and the timing of the various sampling points. Sample (2) taken just before gassing showed abundant fungal mycelium. Upon completion of the gas treatment, sample (3) was obtained and found free of live fungi. It appears that the lack of growth in sample

(3) reflects a definite change in the fungal population when the relative position of samples (1), (2), and (3) is considered. Sample (4) five days after treatment showed no fungal growth. About two weeks after treatment, five more samples (6), (7), and (8) were taken and showed growth. Examination of the hyphae showed no clamp connections, indicating that the organism present was either a species other than Poria carbonica or a primary monokaryotic mycelium of that Basidiomycete. Samples (10), (11), and (12) were obtained six weeks after treatment and all these showed growth of Poria carbonica. The pattern of growth was quite different according to the sample: sample (10) showed no growth on the wood core itself, only on the agar adjacent to the wood, thereby suggesting some residual effect of the methyl bromide. Samples (11) and (12) showed growth on the wood, but primarily on that portion of the core farther away from the surface (Figure 21). Since the methyl bromide was penetrating from the surface it would suggest that the residual effect was affected by the distance from the point of fumigant entrance. This conjecture is reinforced further by the fact that sample (10) came from an area immediately below the point of  $\text{CH}_3\text{Br}$  release.

The vaporization of  $\text{CH}_3\text{Br}$  presented some difficulty: unless the fumigant was released very slowly it tended to rush out of the can in liquid form rather than as a vapor. The occurrence of liquid inside the plastic tent could be observed readily by the sudden formation

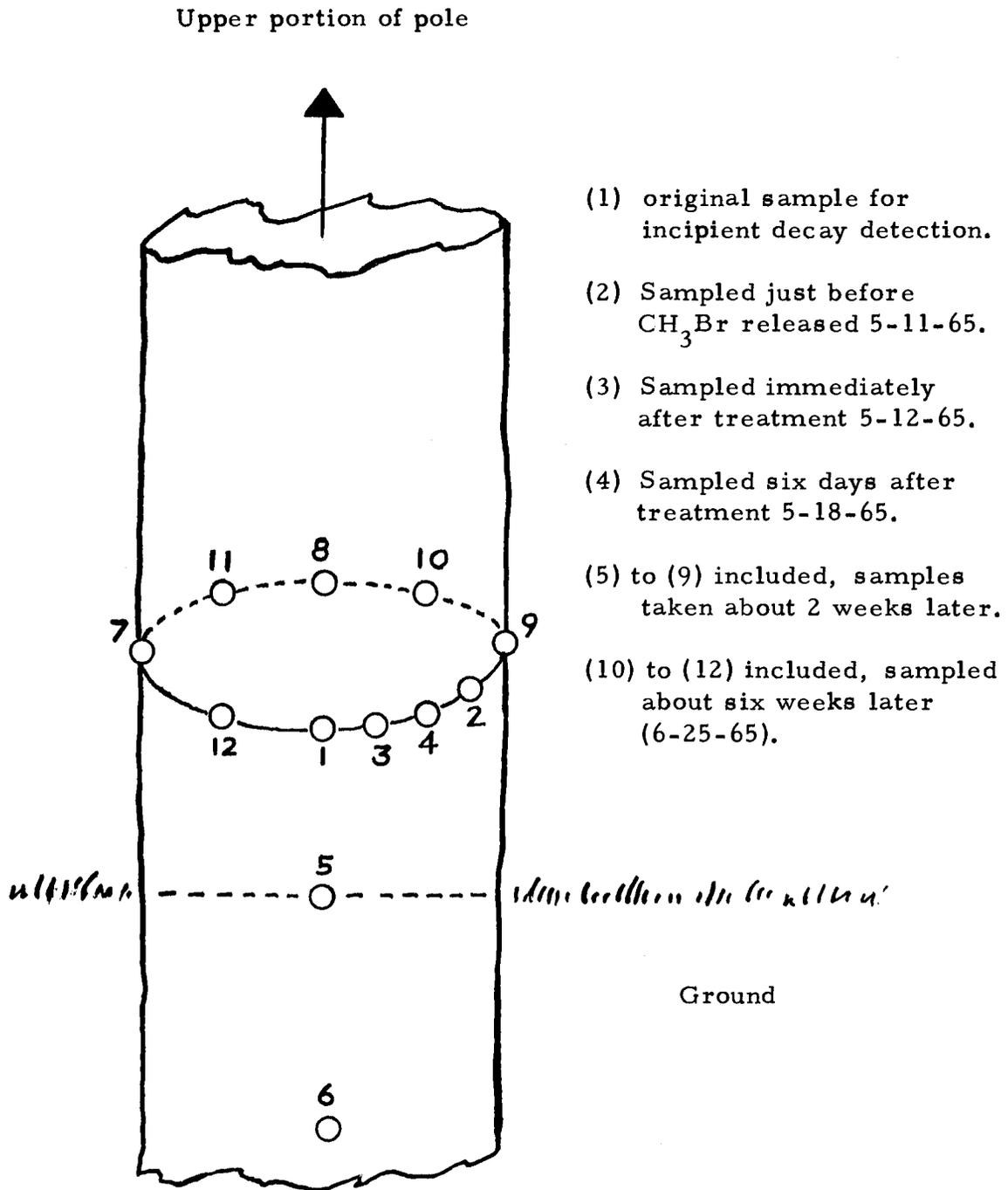


Figure 21. Sampling pattern on pole 14E16 before and after treatment with methyl bromide.

of tiny ice crystals on the outside of the plastic, following the pattern of the liquid methyl bromide in its rapid endothermic change of state. This leaves some questions as to the homogeneity of the distribution of the material over the surface of the pole portion undergoing treatment as well as the radial distribution of the fumigant within the wood.

The vaporization problem was solved in later application by the immersion of the  $\text{CH}_3\text{Br}$  container in a bucket of warm water.

The apparent inconsistency between the results obtained with methyl bromide in the vault and in the field may be due to the reaction of the methyl bromide with the coal tar derivative used for preservation purposes. In the vault a large excess of gas was available compensating for any loss in the system (LeChatelier's principle) which might occur as a result of such reaction. In the field, no such excess prevailed, resulting apparently in only partial destruction of the fungus. Its relatively rapid recovery stresses the importance of as thorough a kill as possible, as well as the value of residual effect from a gas treatment. A very recent sampling, 11 months after treatment, showed some live fungi, but still no occurrence of visible deterioration. Yet as little as two years of incubation is needed from the time of inoculation until advanced decay pockets develop when no substance is present in the wood.

#### Field Test of Multi Gas Diffusion on Used Poles Out of Service

The poles treated in this fashion were all infected with Poria carbonica (Poles No. 6K, 6L and 8E) or miscellaneous fungi (1K, 7F, 7E). Immediately after treatment the poles were sampled again in

the ground line area: pole 7F showed atypical Poria carbonica growth; all others were free from fungi. Because of driving rain during the sampling of pole 7F, difficulty was experienced in maintaining the pole surface free from contamination; that plated sample showed some growth. The pole 7F was sampled twice more with one boring on each side of the original one. No growth was found in these samples.

Upon completion of the fumigation period a beige powder was found on the surface of the poles and on all flat surfaces of the heater. This compound was given qualitative tests by Dr. Molyneux for presence of bromine and ammonium. Both tests were positive. The melting point was not seen, but decomposition seemed to occur at about 225°C. Tetramethylammonium bromide decomposes somewhat above 230°C. The beige compound obtained was readily soluble in water. Its synthesis appears to have been speeded up by heat as its concentration increased inversely with distance from the heater, with a maximum concentration on the heater itself. Concentration decreased from the mid-point of the poles to complete absence at the opposite extremity.

Sampling after about six months of incubation outdoors failed to yield live Poria carbonica after the usual culturing on malt extract agar.

## DISCUSSION

Penetration

The main difficulty in the "in service" treatment of poles is similar to that experienced in the treatment of new poles, but more acutely so: how to achieve penetration of the fungicidal agents within the heartwood, at least to sufficient depth to provide a sound wood shell of about two inches on the periphery of the pole. It is particularly in those poles where the original treatment was unsuccessful in penetrating to sufficient depth that the in service treatment is needed. The present preservation technique is essentially the same as that developed by Bethel in 1838, relying on solutions often of coal-tar creosote, applied under pressure for penetration of the wood. Water solutions have been used also in a number of instances. It appears, however, that these approaches were heavily handicapped by the relative dimensions involved. In order for a substance to penetrate radially through wood within a matter of hours and without major increase of kinetic rates of the reactants involving drastic pressure or temperature increases, small high velocity molecules are required. The maximum molecular speed available is found in the gaseous state, obviously. What is meant by "small molecules" here deserves some careful consideration as it was a decisive factor in the selection of gases made for the treatment of poles.

The major avenue of radial penetration available to a gas molecule attempting to diffuse from an area of high concentration outside the pole to an area of low concentration for that particular gas at the center of the pole, is the bordered pit linking tracheids longitudinally. The distance between the edge of the torus membrane and the edge of the pit is such that many gas molecules could diffuse in and out simultaneously. However bordered pits are often occluded as a result of drying, thereby choking off appreciably this pathway. Krahmer and Côté (1963) have shown that in some cases limited openings remain available in spite of the occlusion. Pores and capillaries have been reported in torus membranes and cell walls respectively, but neither their existence nor their exact size has been demonstrated clearly. Fungal "bore holes" are obvious on photomicrographs of wood cell walls infected with Poria carbonica. All these various openings facilitate penetration of the gas molecules. However, for the selection of an effective gas, the less advantageous conditions must be taken into consideration. A single live hyphal fragment left in a single tracheid in the section treated would suffice to reinoculate the entire pole unless extensive residual effect from the chemical treatment occurs in the critical wood portions. These conditions may be represented by the tracheid lacking all the qualifications listed above and with every bordered pit thoroughly occluded.

Sound cell walls, though quite strong mechanically, do not offer

an extremely tight molecular structure. The three major constituents, cellulose, hemicellulose and lignin, vary in their relative concentration depending on the cell wall layer (Figure 22). Each of these constituents has its own more or less regular molecular structure and intermolecular pattern, resulting in an overall arrangement anything but homogeneous. Hermans (1949) estimates that as much as 20 percent of the dry cell wall volume is air space. If the limiting size opening is sought in this array, it should be found within the most highly organized structure, particularly the cellulose in its crystalline form. Preston (1965) reports a 7.9 Angstroms spacing in one dimension, 8.35 Å in the second, and 10.3 Å in the third between the cellobiose units of crystalline cellulose.

This dimension of 7.9 Å was considered then as the limiting size orifice that the fungicidal gas molecule should be able to enter. In addition to the orifice size an allowance must be made for the frequent molecular collisions in gas causing variations from a straight path. Ultrafiltration studies made on small particles show that as the size particle decreases, the particle diameter to pore diameter ratio decreases, as shown in Table VI.

These figures do not provide a precise answer for pores of 10 Å or 1 millimicron but they provide some guidelines to evaluate the upper limit of the size particle which may be expected to enter an opening of that size: if a 10 millimicron opening will not allow a particle

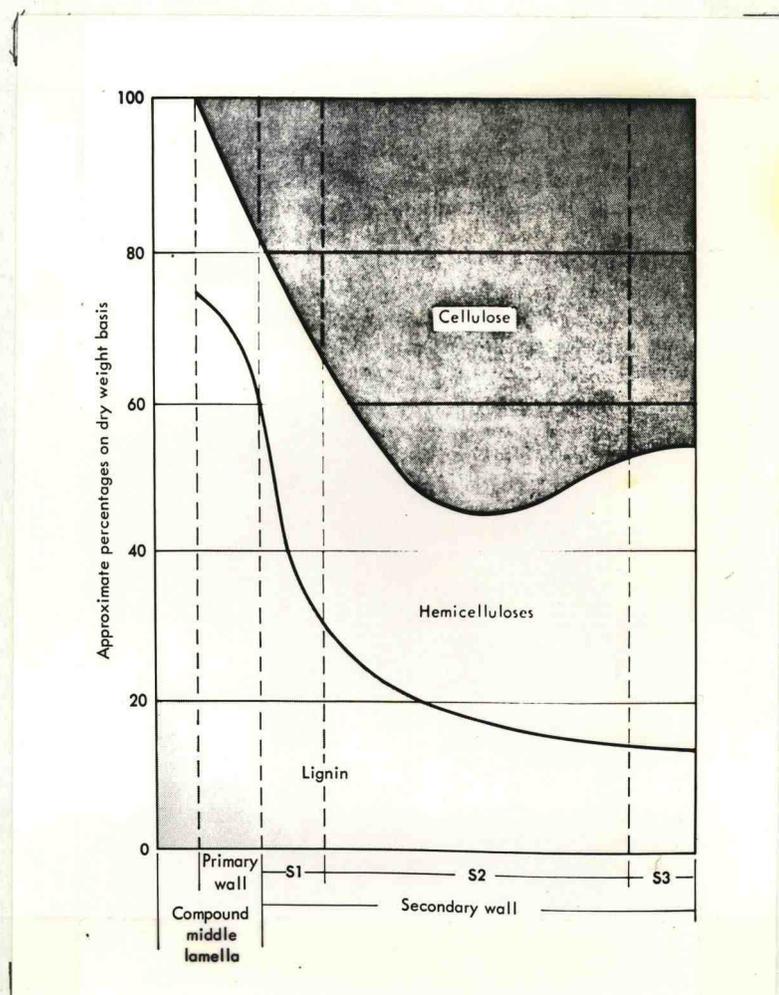


Figure 22. Distribution of the principal chemical constituents within the various layers of cell wall in conifers, according to Drs. A. J. Panshin and C. DeZeeuw (1964)

Table VI. Effect of decreasing pore size on particle-to-pore ratio for penetration

Average Pore Diameter in millimicrons	Particle-Diameter; Pore-Diameter Ratio
500-1000	3:4 to 1:1
100-500	1:2 to 3:4
10-100	1:3 to 1:2

N. B. In this case Brownian movement rather than intramolecular collisions is involved. Because Brownian movement itself is apparently due to molecular bombardment of the particles, the effect of collisions on the particle-diameter: pore-diameter ratio should become increasingly greater as the particle considered approaches molecular dimensions.

of more than 5 millimicrons to pass through, a 7.9 angstrom opening is not likely to pass particles much over 4 angstroms. This size then was considered as the maximum suitable for the purpose.

Table VII presents the relative sizes of various gas molecules known to have at least some fungicidal properties. The first three: formaldehyde, ammonia and methyl bromide had a definite size advantage over the last two, resulting in their selection for experimentation in this study. Formaldehyde was used but not for in service pole treatment. It was applied to ground mycelium prepared for injection into rabbits for antibody induction; its high effectiveness against Poria carbonica is described in the identification section.

#### Methyl Bromide

Methyl bromide (boiling point 3.5 °C) was explored first for in service treatment of infected poles as it is one of the more widely used fumigants for a large variety of substrates and conditions of application: vaults, warehouses, ships, freight cars, soil, etc. Its use in wood has been recommended for usual insecticidal purposes for timber up to 2 in. thick at the rate of 3 lbs. per 1000 cu. ft. for 24 hours at 70°F or above. When the temperature drops to 50°F, which is considered as the lower limit for effective fumigation, an increased dosage up to 4 lbs. is recommended. No specific data were found on the fungicidal properties of  $\text{CH}_3\text{Br}$  or formaldehyde,

on Poria carbonica; hence, first-hand information had to be developed experimentally. A practical dosage of each agent was found highly effective against the fungus tested. In the case of methyl bromide this can be expected from the ionization of the gas molecule either before or after penetration in the hyphal cytoplasm:



Besides the acid, the reaction yields another toxic substance, methanol, which has disinfectant properties.

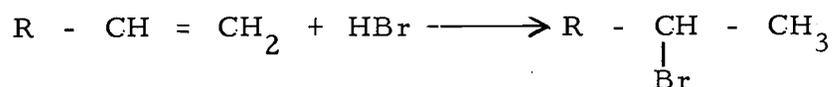
Residual action is also likely to occur through halogenation of the substrate, particularly of hydroxyl groups and alkenes wherein carbon atoms are linked by a double bond. Hydroxyl groups are found in the phenolic part of the lignin or in the aldehyde portion of sugars, besides on the C atoms 2, 3, and 6 (Browning, 1963) of many hexoses:



This enhances the resistance of the compound to microbial enzymes through the dual effect of blocking a reactive group and introducing a toxic atom into the substrate. This latter event is so effective that it is used for the manufacture of fungicides with phenol or quinone as the substrate and chlorine as the halogening agent (Pelczar, 1958).

Alkenes of many forms occur naturally in plant material. Their double bonds react readily with halogen acids, yielding usually a single

compound according to Markownikoff's rule (Cason, 1956):



The fungicidal value of this reaction is of the same magnitude as that mentioned above under alcohols.

### Ammonia

This substance (boiling point  $-33.4^\circ\text{C}$ ) can function in several distinct ways in the treatment of wood: direct fungicidal action, temporary and permanent modification of the substrate when used in the presence of oxygen, and synthesis of fungical compounds in situ when used simultaneously with reactive halides or halogens.

The direct fungicidal action of ammonia has been known for some time. Roistacher, Eaks and Klotz (1955) found that 5,000 ppm twice daily for three consecutive days prevented decay in oranges and lemons inoculated with Penicillium italicum and Penicillium digitatum spores by skin scratch 24 to 30 hours prior to treatment. Further work by the same group (Counther, et al., 1956) established that, as a source of  $\text{NH}_3$ , "ammonium succinate is most promising by virtue of outstanding decay control, probable commercial availability at low cost, favorable toxicology, lack of objectionable odor, hygroscopic nature, and ease of formulation."

Table VII. Relative size of various fungicidal gas molecules

Compound Name	Structure	Type Link- age	Dis- tance in Ang- stroms*	Simplified Structure in Longest Axis**	Maximum Di- mension of Molecule in Angstroms
Formaldehyde	HCHO	C = O C - H	1.21 1.09	H - C = O	2.30
Methyl bromide	CH <sub>3</sub> Br	C - H C - Br	1.09 1.91	H - C - Br	3.00
Ammonia (anhydrous)	NH <sub>3</sub>	N - H	1.01	H - N - H	2.02
Ethylene oxide	$\begin{array}{c} \text{CH}_2 - \text{CH}_2 \\ \diagdown \quad \diagup \\ \text{O} \end{array}$	C - H C - C	1.09 1.54	H - C - C - H	3.72
$\beta$ -propio lactone	$\begin{array}{c} \text{CH}_2 - \text{CH}_2 \\   \quad   \\ \text{O} - \text{C} = \text{O} \end{array}$	C - H C - C C = O	1.09 1.54 1.16	H - C - C = O	4.05
Water ***	H <sub>2</sub> O	H - O	0.96	H - O - H	1.92

\* According to L. F. Fieser and Mary Fieser, Organic Chemistry, D. C. Heath & Co. 1950.

\*\* This simplified linear arrangement does not make allowance for the tetrahedral linkage of C and adjacent atoms; it does meet the chief concern at hand however in indicating the longest possible dimension of that particular molecule.

\*\*\* Theoretically, water should be a gas also at room temperature under atmospheric pressure. The polarity of the molecule results in the formation of hydrogen bonds linking 4 to 8 molecules in liquid water, explaining its behavior so far as boiling point and wood penetration are concerned.

Basidiomycetes, particularly Agaricus campestris, are well known for their sensitivity to ammonia. Not all fungi are equally affected, McCallan and Weedon (1940) reported almost no effect on Glomerella cingulata.

The temporary modification of wood by  $\text{NH}_3$  is due at least in large part to its reversible reaction with cellulose, particularly in its non-crystalline form. Davies (1943) studied the mechanism of the reaction in some detail and suggested that R - OH---N bridges between cellulose and amines were involved. This event results in the swelling of the cellulose and its supporting wood, enhancing the penetration of companion gas molecules. Its reversibility tends to trap the penetrating agent within the wood structure upon completion of the treatment.

The permanent modification of the substrate has been reported to involve the reaction of the sulfhydryl group of thiamin with a  $\text{NH}_3$  molecule thereby altering the susceptibility of this all-important vitamin by fungi (Baechler, 1956; Hartley, 1958).

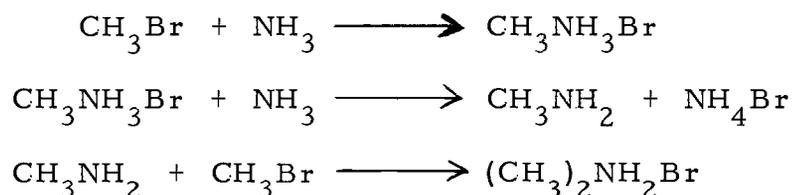
When oxygen is present during the amination process drastic changes appear to occur in the lignin component of the wood. Polymerization may result from linkage of phenyl rings through N and O bridges. The simultaneous oxidation and amination of both natural compounds, particularly in Ponderosa pine bark, and related pure, model compounds was studied in some detail by the writer (1961).

It was found to alter the physical, chemical and biological characteristics of the substrate. The well-known stability of humus is believed due in large part to this type of reaction.

Application to wood preservation has been attempted; Blew (1965) in a current progress report mentions the results obtained in soil-block tests of stakes treated with an ammonium hydroxide solution.

Synthesis of fungicidal compounds in situ when used simultaneously with reactive halides or halogens may be extremely useful for residual protection of the treated wood.

When ammonia and methyl bromide molecules are allowed to collide the following sequence of reactions can be expected (Fieser and Fieser, 1950):



Repetition of the successive amination and bromination builds up the alkyl radical of the compound until  $[(\text{CH}_3)_4\text{N}]^+\text{Br}^-$  is formed. This compound is typical of the well-known quaternary ammonium salts. Their germicidal properties are widely recognized and used commercially for many purposes as their relative stability and versatility make them exceptionally effective; their cost, however, is limiting.

The beige compound actually recovered from the multi gas diffusion treatment of the poles is a water soluble ammonium salt containing bromine and of relatively high molecular mass.

Multi gas diffusion treatment of poles in service, as well as any other wood structure where penetration of the fungicidal agent presents a problem, appears promising particularly with the use of such gases as  $\text{CH}_3\text{Br}$ ,  $\text{NH}_3$ ,  $\text{O}_2$  and other halides or halogens ( $\text{CH}_3\text{Cl}$  for instance). It results in a variety of fungicidal or fungistatic reactions, thus making the circumvention of the treatment by a mutant or adapted form of a wood-destroying fungus highly improbable.

## CONCLUSION

The destruction of Poria carbonica or similar fungi within the heartwood of Douglas-fir poles in service appears feasible at atmospheric pressure when a mixture of suitable gases are used, if provision is made for the confinement of the gases to the area treated. Additional effort is needed to evaluate the optimum kinds, concentrations and sequences of gases to be used, their method of application and the residual effect of the treatment.

## SECTION III - CONTROL: B - BIOLOGICAL CONTROL

## INTRODUCTION

Biological control is in Garrett's words (1965):

. . . any condition under which or practice whereby, survival or activity of a pathogen is reduced through the agency of any other living organism (except man himself), with the result that there is a reduction in incidence of the disease caused by the pathogen. Biological control can be brought about either by introduction or by augmentation in numbers of one or more species of controlling microorganisms, or by a change in environment conditions designed to favor the multiplication and activity of such organisms, or by a combination of both procedures.

Biological control then does not include the application of antibiotics, since they are no longer part of a living organism, though living organisms used for biological control may well secrete antibiotic(s). Some work with antibiotics is included in this section nevertheless since it was more convenient to work with preparations of this type rather than with the various organisms known to synthesize these substances. The underlying assumption was that if no result was obtained from the antibiotics, none would be available for the corresponding live sources. Application of the antibiotic cycloheximide has been valuable for the control of blister rust on live western white pine (Lemin, Klomprens and Moss, 1960; Moss, 1961) and related applications may have value for short term control of heartwood decay in poles. This has not been studied here.

Biological control is generally much more complex to secure than chemical control in that it requires more detailed knowledge of a given ecological situation, but it offers distinct advantages:

1. No lingering residues causing undesirable side effects.
2. On occasions, much lower application cost.
3. More flexibility to cope with genetic changes in the pathogens.

These advantages have been observed more directly so far with animals, particularly insects rather than with plants, yet it may be that their application will eventually turn out to be just as beneficial in that latter kingdom, where no immunological reactions prevent an intimate contact between host and live commensal.

Successful biological control with plants has been primarily indirect so far, in that the environment, usually the soil, is modified to encourage the activity of antagonists already present rather than the direct introduction of desirable organisms.

## REVIEW OF LITERATURE

The lack of precedent in this immediate area invites a brief presentation of related activities.

The infection of potatoes with Actinomyces scabies, now known as Streptomyces scabies, and similar species, causing potato scab can be controlled by green manuring encouraging the development of Actinomyces praecox, now called Streptomyces praecox, and other saprophytic antagonists of A. scabies (Millard, 1923; Millard and Taylor, 1927; Sanford, 1926). The same cultural practice aids the control of the take-all fungus in wheat, Ophiobolus graminis through the increase in population of antagonistic saprophytic species (Lal, 1939). An increase in carbon dioxide in the soil from the enhanced respiration of the microbial population resulting from the addition of the readily metabolized carbohydrates reduces the extent of that disease. Other diseases controlled by the addition of organic residues, include cotton and strawberry root-rot (Mitchell, Horton and Clark, 1941; Hildebrand and West, 1941).

Bollen and Glennie (1961, 1963) made extensive studies on the control of red stele fungus disease in strawberries with Douglas-fir sawdust and bark additions to the soil. Sawdust mulches were found to induce lower soil temperature favoring the disease in wet soil if the fungus is present. Ground whole Douglas-fir bark without added nitrogen

had little or no effect. Ammoniated bark at 4.3 percent nitrogen lowered the incidence to about 50 percent, while hot-water-extracted bark nearly eliminated the disease; yet ammoniated hot-water-extracted bark was comparable to the ammoniated plain bark.

The more direct approaches to pathogen fungus control by antagonistic microbial species were explored primarily by Russian workers: Naumova (1939) reported on the effect of seed treatment with bacteria for the control of summer wheat seedling fungal pathogens. This is somewhat related to the inoculation of legume seeds for the establishment of rhizobia nodules, in that proper treatment of the seed results in the distribution of the desired organisms along the root hairs, as demonstrated by Nutman (1965). Closer to the problem of Douglas-fir poles, Negrutskii (1963) studied the control of Fomes annosus in pine forests with Trichoderma lignorum. The degree of antagonism between these two species was evaluated in petri dishes containing Capek's medium.

Inocula of T. lignorum spores were used to spray pine stumps thereby preventing the spread of F. annosus.

Other experiments of this type were performed with Peniophora gigantea by Negrutskii and also by Rishbeth (1963). However that organism is a wood destroyer so that its application to wood structure would not be desirable.

In the case of Trichoderma lignorum, starch is the main

component of the wood substrate used so that little damage should result to the mechanical strength of an inoculated wood structure. This was the conclusion reached by Lindgren (1952) when he inoculated the related, if not identical, species T. viride in fence post material in order to improve its permeability.

## MATERIALS AND METHODS

The approach used here is somewhat different from any of those mentioned above in that the biological control agent is introduced in the internal environment of the pole to be protected rather than in some portion of the pole external environment. The biological control agent is expected to develop within the pole heartwood, using it as a growth substrate in such a way as to leave its critical properties, i.e. mechanical strength, unharmed.

Two different methods were explored for this purpose: bacterial spores application on the surface of the poles and inoculation of fungal spores within the heartwood of poles.

A preliminary experiment was performed to assess the potential value of standard antibiotics against Poria carbonica with the intention of testing the live source of the effective antibiotic for adaptation to the environment of Poria carbonica.

### Standard Antibiotics

Difco antibiotic discs with the following antibiotics were applied to malt extract agar plates inoculated at the center with a Poria carbonica covered agar block:

streptomycin	2 mcg.	penicillin	2 units
chloromycetin	5 mcg.	tetracycline	5 mcg.
kanamycin	5 mcg.	novobiocin	5 mcg.

erythromycin	2 mcg.	neomycin	5 mcg.
coly-mycin	2 mcg.	oleandomycin	2 mcg.

### Bacterial Antagonists

Spore forming bacteria are known to grow in certain timber species creating various difficulties in the seasoning process and during wood storage including sinkers (Knuth and McCoy, 1962; Knuth, 1964) and irregular permeability of pine sapwood (Elwood and Ecklund, 1959). Strains of these bacilli secrete antibiotics: subtilin and polymyxin. These organisms are found occasionally in hemlock samples processed in the Laboratory, but none had been reported in Douglas-fir. Taking into consideration the fundamental principles of genetics and such practical corroborating evidence as the development of DDT resistant houseflies, hydrocyanic acid resistant aphids, penicillin resistant staphylococci, etc., it was felt that a strain able to prosper on Douglas-fir should be available through the automated scanning of a sufficiently large population of these bacilli. Repetition of the process would select out an anti-Poria carbonica variety among the Douglas-fir adapted cells. The spores of that culture would then be sprayed on the poles so that germination of Poria carbonica contaminants would be accompanied by the germination of the antagonistic bacterial spores.

Spore forming bacteria were isolated from wetwood streaks in

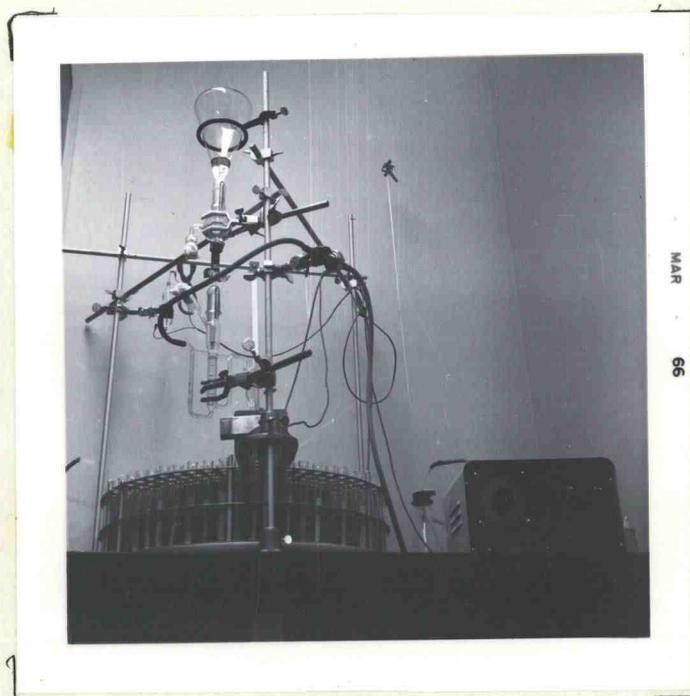


Figure 23. Chemostat and fraction collector synchronized for the automatic scanning of large bacterial populations. The continuous culture unit provides a suitable environment for the intense reproduction of the normal strain, while the individual tubes provide a different environment. Only those bacterial cells with a broader enzymatic make-up than the normal population are able to survive in the tubes containing Douglas-fir wood wedges.

hemlock secured through the cooperation of Mr. C. J. Kozlik. The isolates were grown in malt extract broth in a 250 cc shake flask, then in a chemostat synchronized with a fraction collector holding 18 x 150 mm tubes. Five ml increments were collected in each tube during a 20 minute time period. Each tube contained a wedge of heartwood about one by one by seven cm long: hemlock in one tube out of five in the normal sequence, and Douglas-fir in the others. The hemlock wedges functioned as controls on the physiological stability of the culture, while the Douglas-fir wedges provided the selective environment.

The various rates used were based on the normal mutation rates ( $1 \times 10^{-6}$  cells), prototroph incidence among auxotrophs ( $10^{-5}$  mutants), bacterial population density in the continuous culture unit (about  $10^7$ /ml), as discussed in a previous report (Ricard, 1962).

#### Douglas-fir Adaptation

The occurrence of Douglas-fir adapted cells was revealed presumptively by a continued cloudiness of the spent broth delivered in the tubes. These fractions were diluted with about 15 ml of distilled water causing the wood wedge to float within the tube liquid.

### Confirmed Douglas-fir Adaptation

Confirmative evidence of Douglas-fir adapted cells was secured by further growth of the bacterial cells in the system, particularly alongside and within the wood wedge causing it to sink, in the traditional manner of the infected logs known as sinkers.

### Presumed Antifungal Activity

Presumptive antifungal activity from these bacteria was demonstrated by absence of the fungal matt normally formed on the surface of the tubes saved for the confirmed test. The two-week incubation period required was carried out in tubes without plugs to prevent contamination. As a result, the ubiquitous fungal species would inoculate the surface of the tube contents, forming a mycelial pellicle on the surface of the tubes. The occasional absence of such mat was readily noticed and its cause tested by further inoculation with two or three loopfuls of fungal spores from an adjacent tube.

### Confirmed Antifungal Activity

The lack of growth of these inoculated fungal spores was considered to be evidence of confirmed antifungal activity to be investigated further with a pure culture of the prominent bacteria present in the tube.

### Fungal Antagonists

No attempt was made to develop a strain specifically for antagonistic effect, though systematic selection of the more actively antagonistic cultures was practiced.

During routine detection studies, a fungus, F-y, antagonistic towards Poria carbonica was found and isolated. F-y was then studied in some detail, using the same methods as in other aspects of the work augmented with specific techniques. These methods included: moisture determination in the poles, toughness tests, cultures on malt extract agar with and without sound wood, thin section examinations and photomicrographs, agar colonies examination with epillumination and photographs, inoculation in poles, separation of the pigment from the culture medium and also separation of its various components by paper chromatography.

#### Effect on Tracheid Wall

The effect of F-y growth on the tracheid wall was studied by placing steamed Douglas-fir veneer sections on a malt extract agar plate inoculated with the usual mycelium-covered agar block. The veneer sections, 10 x 3 x 70 mm, were steamed under atmospheric pressure in the autoclave for 20 minutes before use to eliminate surface contamination. The F-y covered the section entirely, though

lightly, within a week; after another two weeks sections were prepared as described in the Identification section under Materials and Methods, or used for pole inoculation in the field.

#### Examination of Colonial Characteristics

Malt extract agar plates were inoculated with 10 x 5 mm agar block overgrown with F-y and examined at 50X to 100X under a Zeiss light microscope equipped with epi-illumination condensers and objectives, as well as with standard illumination. Photomicrographs were taken at various intervals with a Zeiss camera and adapter.

#### Pigment Extraction and Separation

When cultured on agar medium, F-y often diffused its yellow pigment beyond the edge of the mycelium. That portion of the agar, free from mycelium, but pigmented, was cut in strips three to five mm wide and up to 30 mm long. These strips were suspended in distilled water or other solvent and shaken for 30 minutes. The colored water was then filtered off the strips and evaporated at room temperature. Amorphous solids were obtained eventually and placed on the agar in plates inoculated with Poria carbonica for the evaluation of the antibiotic activity.

### Paper Chromatographic Fractionation

The pigmented agar was extracted with methanol and filtered. The colored filtrate was concentrated under mild heating, then a few drops were placed on Whatman No. 1 paper for separation. The components were eluted with 5:2 methanol:water solvent for 2.5 hours with a descending front. Bisdiazotized benzidine reagent was applied to identify phenolic compounds.

### Pole Inoculation

When thin sections of Douglas-fir veneer sticks showed numerous hyphae and conidia in the cell lumina, they were introduced as needed in the openings left in the poles by the removal of increment borer cores and the opening closed with a treated wood dowel. Suitable precautions to avoid contamination were taken throughout the inoculation process.

## RESULTS

### Standard Antibiotics

These substances appeared to have no effect on the growth of Poria carbonica, while an accidental contaminant did, see Figures 24 and 25.

### Bacterial Antagonists

No difficulty was experienced during the isolation and culture of the hemlock bacilli in malt extract broth. In most cases the chemostat effluent cleared up within 24 hours after collection in the Douglas-fir tubes. The bacterial suspension remained cloudy in about two percent of the tubes inoculated from the third day of continuous operation on. Since the collection rate averaged 72 tubes per day, about one to two tubes per day were set aside for further incubation with the diluted spent malt extract broth and fresh Douglas-fir wedge. The number of tubes remaining free from surface growth again averaged about two percent. All the tubes showing presumptive evidence of antifungal activities were confirmed as such upon inoculation of the tube with fungal spores (Figure 26). The contents of these tubes were inoculated in a 250 erlemeyer flask containing Douglas-fir wedges and distilled water. A heat wave which brought the temperature in the laboratory to over 40°C stopped the growth in

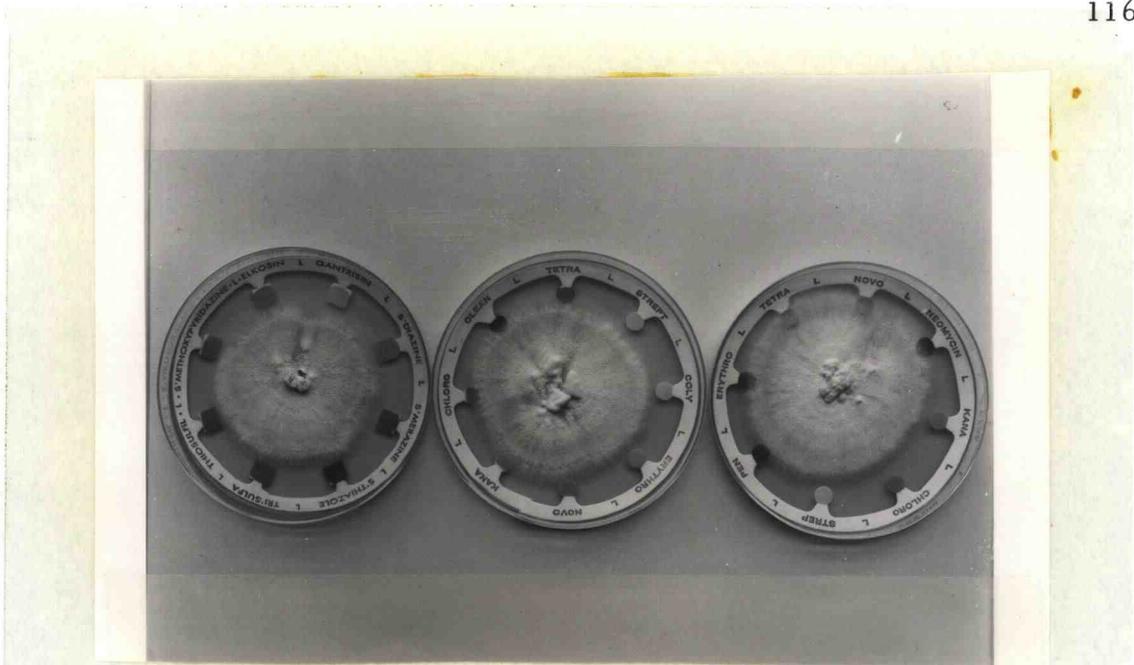


Figure 24. Standard antibiotics and sulfa drugs have no obvious effect on *P. carbonica* growth.



Figure 25. *P. carbonica* is sensitive to certain "wild" antagonist.

the flasks after incubation for 48 hours. By the time the fall weather brought back consistently lower temperatures, the emphasis in biological control had shifted to fungal antagonist.

#### Fungal Antagonist

F-y was detected during routine culturing operations. Its occurrence presented a new pattern in pole fungal infection and as such was examined in some detail. This led to the observation of its antagonistic properties toward Poria carbonica in vitro. Just how useful this property might be in vivo is not known yet though field tests are under way. The following results were obtained through the laboratory cultures of F-y and the examination of the used poles where it occurred naturally.

#### Cultural Characteristics

The most obvious characteristic is its bright yellow, water soluble, pigment, which diffuses throughout the plate after an F-y infected wood core is incubated on a malt extract agar plate. This results in a change in the appearance of the agar medium well ahead of the spreading mycelium. On occasions, the entire plate became yellow when examined after one week in incubation. After maintenance on artificial media for several weeks, F-y frequently loses its pigment producing ability. In the case studied, pigment synthesis

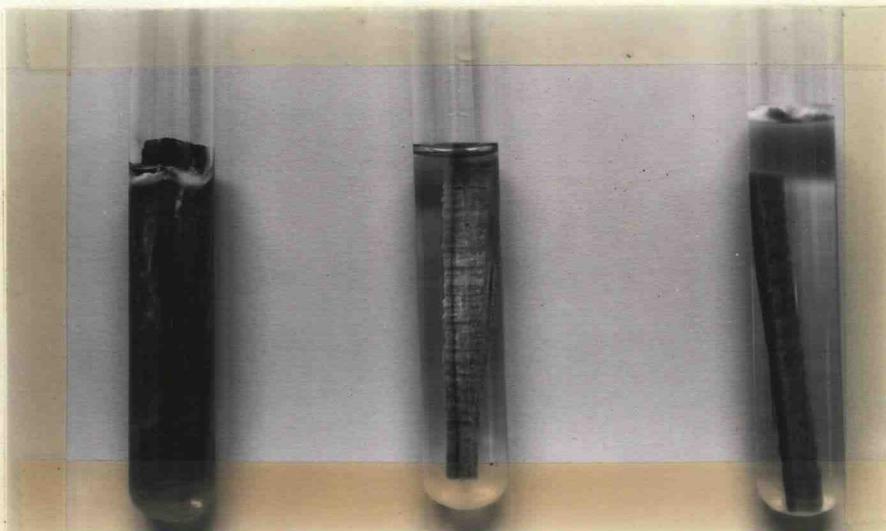


Figure 26. Variation in response to fungal invaders of various bacterial strains adapted to Douglas-fir. All three tubes contain bacteria, the two end tubes have also a fungal pellicle, while the center tube did not support fungal growth in spite of natural and artificial inoculations.



Figure 27. "Wild" and laboratory strains of F-y in presence of *Poria weirii*. Two days after inoculation F-y strains show a variation in pigment synthesis: the strain growing in wood is already producing the pigment, while the laboratory strain at the center of the plate does not. Later on, this latter strain resumed pigment synthesis.

was resumed whenever the culture was returned to a competitive situation, as by simultaneous planned or accidental-inoculation with fungi or bacteria. In every case, F-y released its yellow pigment and also completely overgrew the competing organisms. In one instance a strain was alternatively grown with and without competition; it proceeded to adjust its pigment synthesis accordingly (Figure 27).

All the "wild" strains, directly isolated from a pole, were found to produce the yellow pigment. The aerial mycelium itself, however, seems colorless or slightly grey although the pigment appears to be synthesized by endoenzymes. As the colony becomes older and starting with the original inoculation, another color change takes place, from grey to black. Eventually the entire colony becomes black. This event requires about one month to develop fully.

#### Microscopic Characteristics

At about 100X magnification, the black coloration appears to coincide with the formation of conidia in chains at the tip of hyphae in the aerial mycelium. The hyphae are greyish, abundant, branched, two to four microns in diameter. Spores are oval to round, 4.5-7 microns in diameter, light brown to black in color. The yellow pigment appears to occur first within certain hyphal tips (Figure 28).

Branching hyphae form various patterns ranging from simple curling to complete rings or even ball-like bodies (Figure 29). The

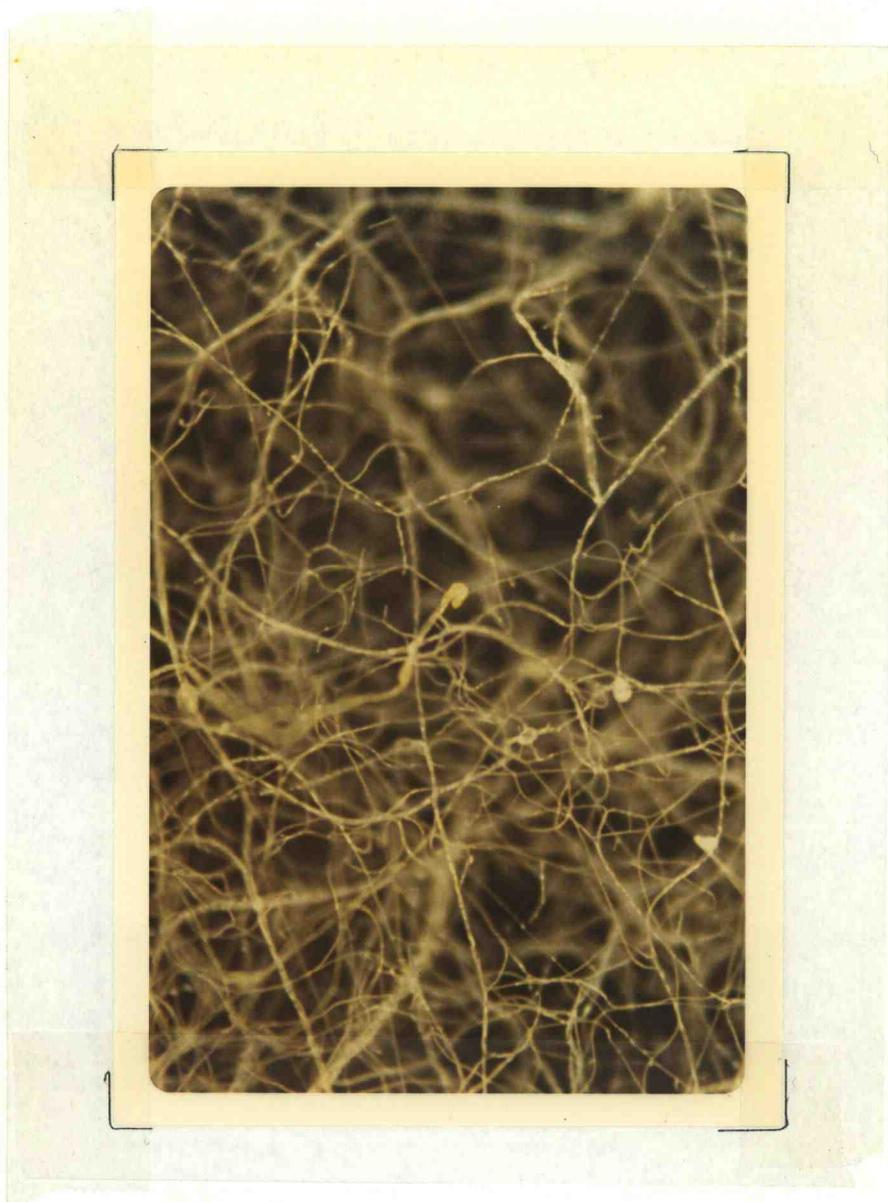


Figure 28. The yellow pigment is synthesized by endoenzymes apparently. It occurs in occasional hyphal tips first, then dissolves throughout the supporting medium.

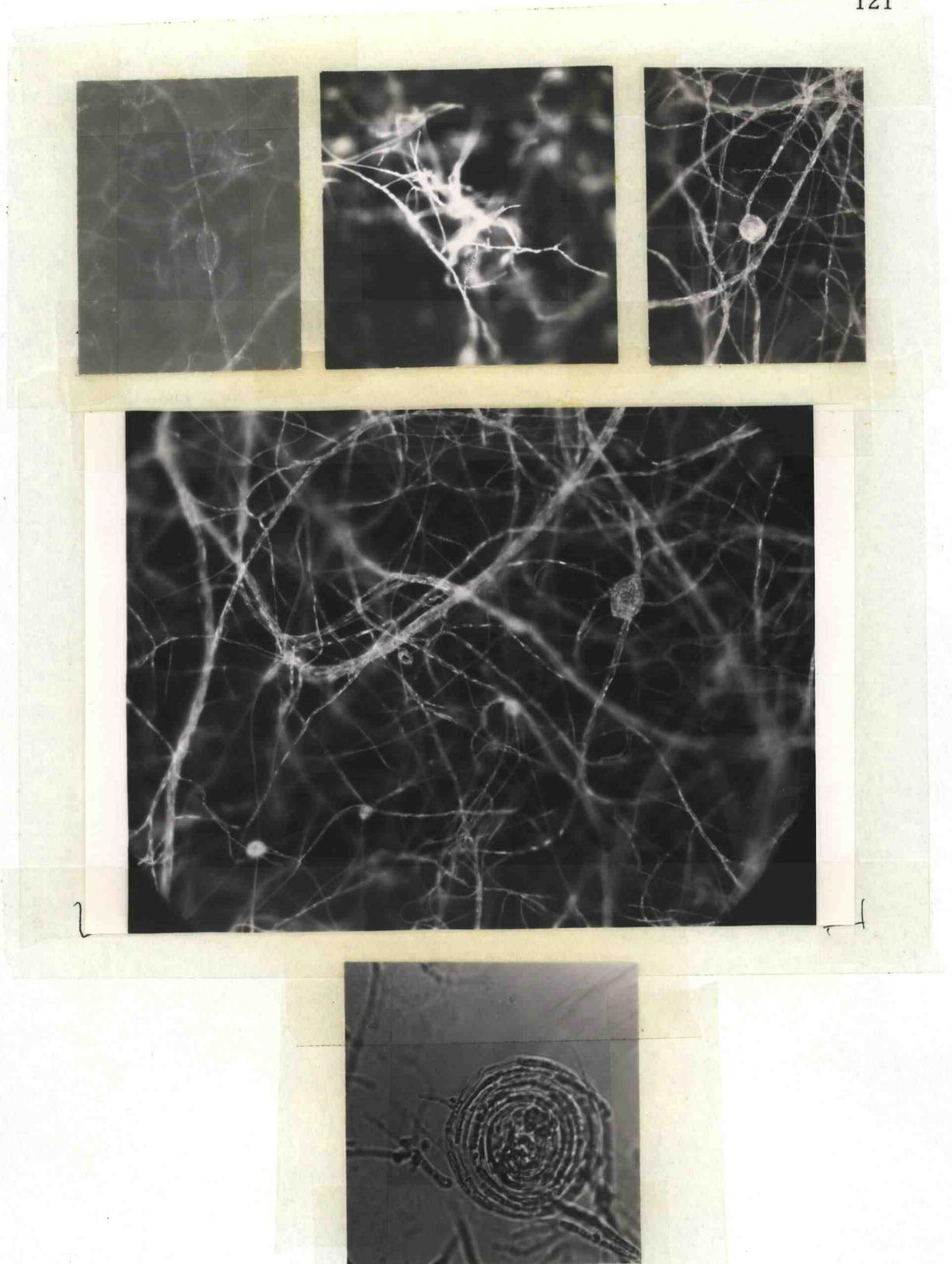


Figure 29. The aerial hyphae of F-y offer a variety of morphological features shown here, at various magnifications.

more elaborate forms were at first taken for zygospores, leading to the tentative classification of the organism with the Phycomycetes. More thorough examination by Professor L. F. Roth and Dr. E. E. Nelson rectified this observation. Dr. Roth indicated that F-y belonged probably to the class of Deuteromycetes and the order of Moniliales, but that it did not resemble any of the better known genera of that group. He added that he had observed previously similar organisms in cultures of forest litter. A culture has been mailed to the USDA Forest Disease Laboratory, Route 2, Box 263, Laurel, Maryland, for further identification.

#### Activity Against *Poria carbonica*

Simultaneous inoculation of plates with actively growing sections of mycelia from F-y and *Poria carbonica* invariably resulted (Figure 30) in the sequence of events described by Negrutskii (1963) concerning the relative behavior of *Trichoderma lignorum* and *Fomes annosus*. Subculture of *P. carbonica* mycelium covered with F-y yielded F-y colonies only.

Field tests are under way with the inoculation of a *P. carbonica* infected pole with F-y permeated Douglas-fir heartwood sticks and F-y infected pole with similar size sticks permeated with *P. carbonica*.

### Effect of F-y on Douglas-fir Heartwood

Direct Visual Examination: Incubation of four groups of two F-y infected pole sections for seven months under four different regimes of temperature and moisture failed to result in any visible deterioration while P. carbonica infected sections under similar conditions showed extensive damage.

Cellular Level Examinations: The results obtained with thin sections of 0.3 by 1 by 7 cm wood sticks permeated with F-y hyphae were reported in the Identification section, under Results. No evidence of tracheid wall lysis was observed, Figure 31.

### Nature of Pigment and Source of Antibiotic Activity

The yellow pigment extracted from the agar medium failed to show antagonistic activity against Poria carbonica when placed next to an actively growing colony in a plate. The colored and colorless agar overgrown by F-y were both extracted and the extracts fractionated by paper chromatography. Both colored and colorless fractions yielded one identical spot but in addition the color extract yielded a second, less mobile compound (Figure 32). All spots appeared to include phenolic groups. Dr. Molyneux has performed this aspect of the work; and he is considering its further pursuit for isolation of the active component and, possibly, structural identification.

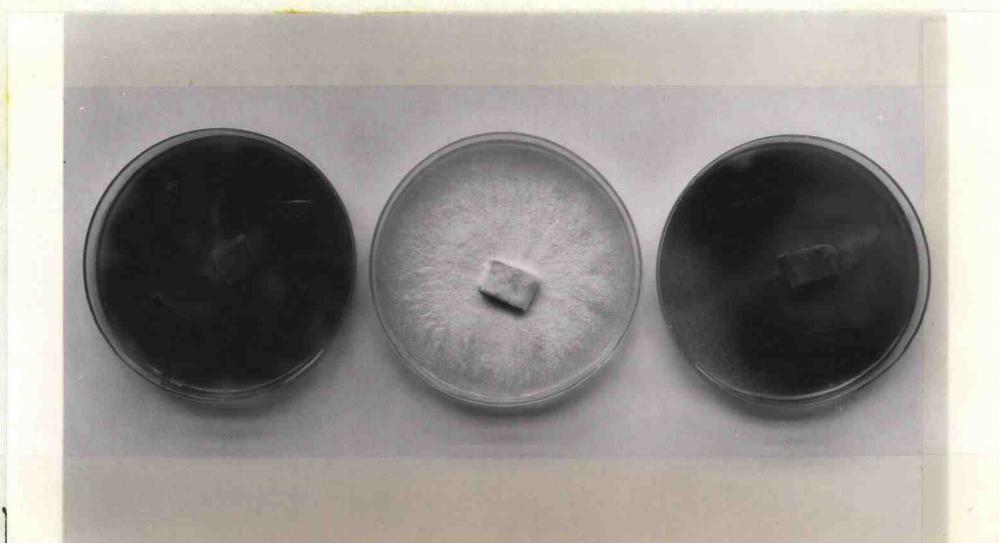


Figure 30. The antagonistic action of F-y toward P. carbonica is unrelated to the relative inoculum size. Both end plates two and four F-y inocula respectively, failed to yield live P. carbonica. In center control plate, P. carbonica growth was unhampered by F-y.

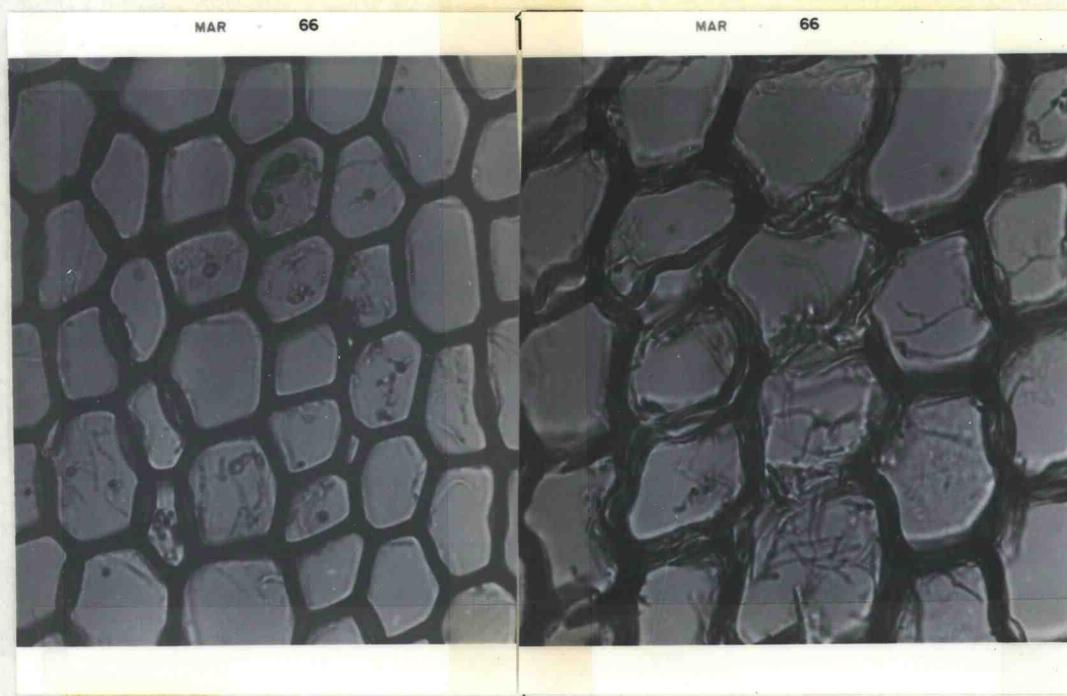


Figure 31. F-y on the left and P. carbonica on the right vary distinctly in their effect on the tracheid wall when crosssections are examined. Both samples were inoculated and incubated in the same fashion.

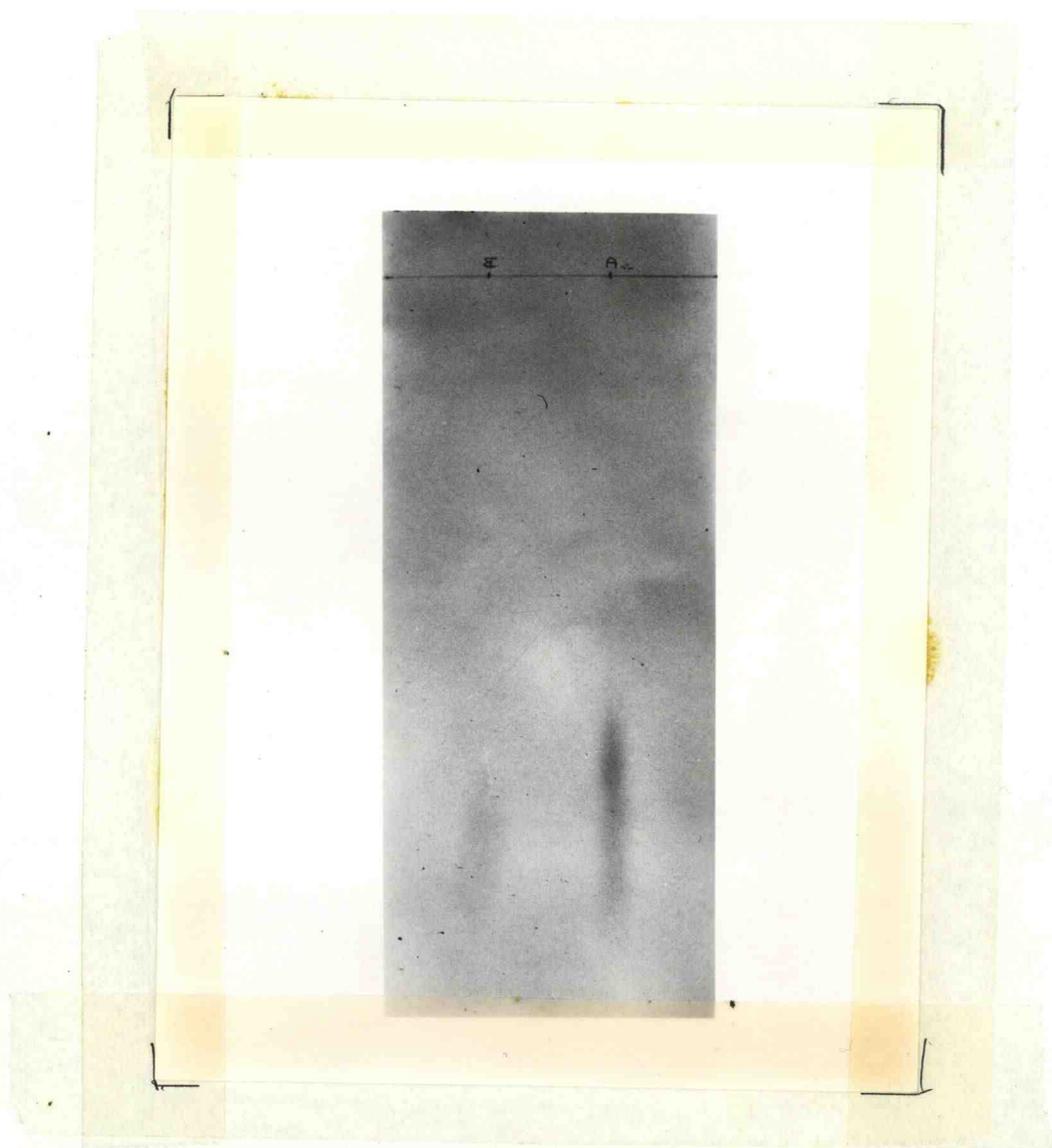


Figure 32. The upper spot is naturally colored and occurs only in the pigmented cultures; the lower spots are found regardless of pigment synthesis. Only the more contrasty lower spots showed on the photograph.

## DISCUSSION

The first concern was to verify the presumptive evidence detected in the poles that F-y and Poria carbonica are not compatible. This was quite obvious in vitro using the same sort of competitive situation between F-y and Poria carbonica, as Negrutskii had used for Trichoderma lignorum and Fomes annosus.

The second question was concerned with the effect of F-y on the mechanical properties of the pole where it had developed. Preferably evidence should be secured at the systemic, cellular and molecular levels to support a definite conclusion. Only the first two areas were explored, but both yielded the same sort of evidence: no visual damage to the wood structure. Nutritional studies measuring utilization or growth on pure compounds, oxygen uptake or carbon dioxide evolution have not yet been attempted.

Field inoculation should provide useful information if F-y develops readily within the poles and brings about the desired results on Poria carbonica. Difficulties in the field inoculation of poles may emphasize the necessity for nutritional studies on the organisms. Current plans call for the inoculation of at least 10 poles this spring and 100 next spring.

Should F-y continue to show the needed characteristics for effective control of Poria carbonica, it might prove to be more

satisfactory than bacterial spores through superior distribution and metabolite transportation system. Bacterial spores may be distributed in extremely high concentrations over the air exposed pole surface and provide protection so long as the initial infection focus occurs in that area. However, should the infection start below ground level, little benefit would be obtained through the bacterial spore application. In the case of F-y, penetration throughout the wood takes place rather rapidly followed by spore formation in many cell lumina, so that the entire pole zone where moisture is adequate for fungal growth may become permeated with the Poria carbonica antagonist. Assuming that pigment and antibiotic synthesis mechanism are similar, invasion of a pole permeated with F-y hyphae would very likely trigger the synthesis of antibiotic substances. This response would provide protection in depth against invading wood destroyers.

Further work is planned on the separation of the compounds responsible in the antibiotic properties of F-y, the identification of their molecular structure and their effect in mammals.

## CONCLUSION

The standard antibiotics tested had no effect on Poria carbonica.

Strains of hemlock heartwood inhabiting bacteria can be selected for ability to grow in Douglas-fir and release antifungal metabolites.

A fungus occurring naturally in Douglas-fir heartwood of poles has definite antagonistic properties against Poria carbonica. This antagonistic fungus appears to have no detrimental effect on the mechanical properties of the wood when examined at the systemic or cellular level.

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## APPENDICES

Appendix A. Morphological characters used in the taxonomy of species of Poria, according to Dr. J. L. Lowe

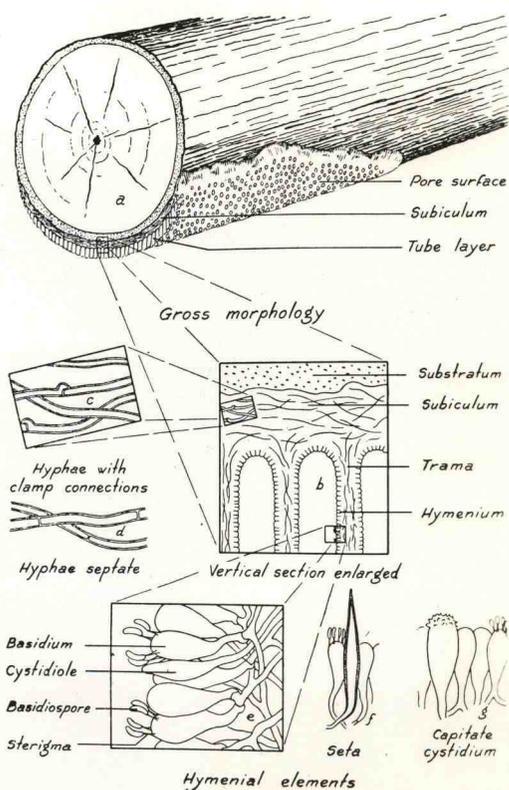


Figure 33. The rectangular areas are successive enlargements of the small rectangle shown in (a) on the log.

Appendix B - Poria carbonica Overholts, according to  
Dr. M. K. Nobles (1948)

Growth Characters

Growth moderately rapid to slow, plates covered in four to six weeks. Advancing zone even, hyaline and appressed in zone up to 1.0 cm. wide. Mat white or with tinges of "pale chalcedony yellow" (9.0 Y 4.5/8.3), appressed, downy to woolly-felty, after three to four weeks producing raised balls of mycelium with cottony or velvety surfaces, along radii or scattered. Reverse unchanged. Odor of apples. On gallic and tannic acid agars no diffusion zones, colony 2.0-3.0 cm. diameter on gallic acid agar, no growth on tannic acid agar.

Hyphal Characters

Advancing zone: hyphae hyaline, nodose-septate, 1.5-3.0 (-6.0) microns diameter. Aerial mycelium: (a) hyphae as in advancing zone; (b) conspicuous much-branched hyphae, the branches usually attached at right angles and frequently rebranched, the walls slightly thickened and rigid, lumina fairly broad and apparently empty. Aseptate, 3.0-6.0 microns diameter; (c) conidia numerous, borne singly at the tips of branches, which are usually narrower than the main hyphae, about 1.5 micron diameter, thin-walled,

broadly ovoid, slightly truncate at distal end, pointed at attached end, 7.0-9.0 x 4.5-7.0 microns; (d) chlamydospores numerous, intercolony and terminal, walls slightly thickened, broadly ovoid. 9.0-16.5 x 7.5-12.0 microns. Submerged mycelium: (a) hyphae as in advancing zone; (b) chlamydospores as in aerial mycelium; (c) crystals numerous, octahedral.

Type of Rot: brown rot of western coniferous trees.

The key patterns for P. carbonica, showing chlamydo-pores, conidia and the much-branched rigid hyphae known only in this species, stand alone in the key and the species is readily identified.

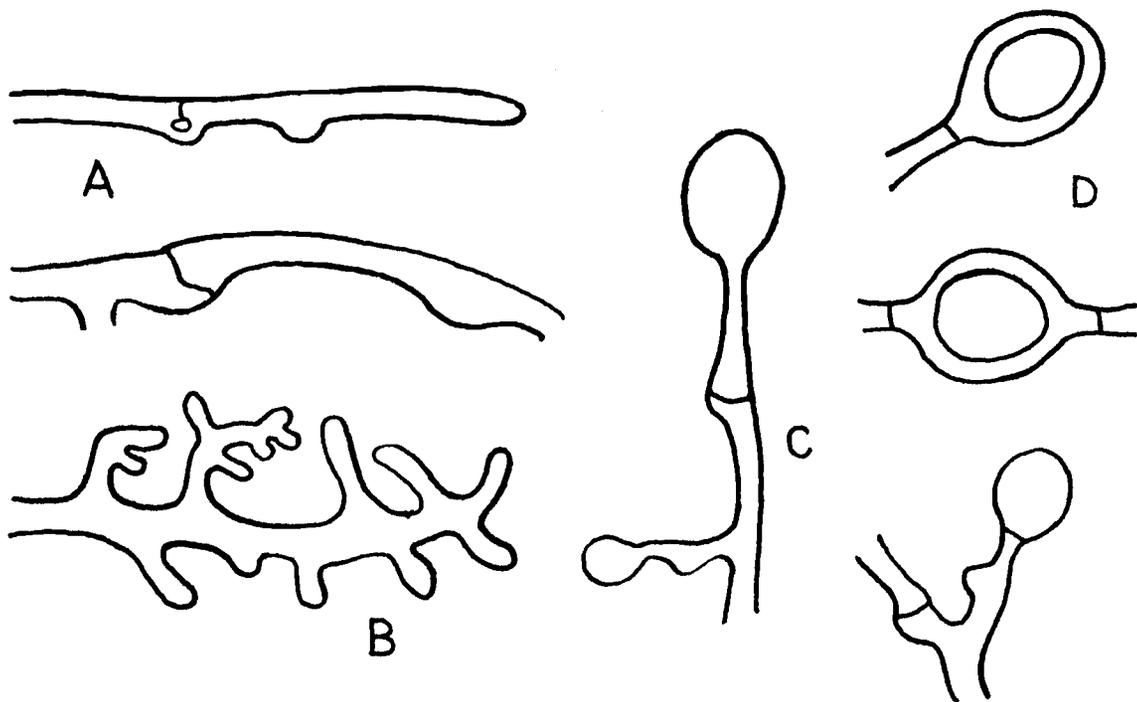


Figure 34. Cultural characteristics of Poria carbonica:  
 A-Hyphae from advancing zone, B-Hyphae from  
 aerial mycelium, C-Conidiophores and conidia,  
 and D-Chlamydo-spores.