FUNGICIDAL TOXICITY
OF
CERTAIN EXTRANEOUS COMPONENTS
OF
DOUGLAS FIR HEARTWOOD

by

ROBERT W. KENNEDY

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ABSTRACT

The heartwood of Douglas fir (Pseudotsuga menziesii (Mirb.) Franco) is known to be relatively resistant to attack by wood-destroying fungi. Previous investigations on other species has established various heartwood extractives as the primary deterrents to decay. Several extraneous fractions from Douglas fir were isolated and evaluated for fungicidal activity in order to determine the precise factor influencing the durability of this species.

An acetone, ether and water extraction of Douglas fir heartwood meal provided five separate components, namely: a dihydroquercetin, free acid, neutral, phlobatannin and carbohydrate fraction. A bioassay of these materials was made using Fomes annosus (Fr.) Cke., Lentinus lepideus Fr. and Poria incrassata (B.&C.) Curt. as the test fungi. Both a cellulosic and a non-cellulosic substrate were employed. Small wood blocks from which certain extractives had been removed were used for the cellulosic substrates, whereas malt agar impregnated with varying concentrations of the extraneous materials represented the non-cellulosic media. The degree of effectiveness of each component as a fungicide was expressed numerically.

Dihydroquercetin was found to be the most potent fungicide, completely inhibiting growth of the most sensitive fungus at a concentration of slightly less than 0.5 per cent.

This value compares favorably with experimental results previously reported with phenolic extractives of the genus Pinus. On the basis of these data, timber selected for its high dihydroquercetin content could be expected to have an extended service life when used under conditions favoring decay. The possibility of breeding highly resistant genetic types is also discussed briefly.

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I. INTRODUCTION

It has been estimated that nine per cent of the total annual drain on the forests of the United States is used to replace wooden timbers in service that have been destroyed by fungi. A further drain of six per cent is due to fungal, insect, drought and wind damage in the forest itself (6). Comparable statistics for Canada are unavailable, but undoubtedly approximate those of the United States.

It is important that the factors influencing the natural decay resistance of our major timber species be understood, so that those woods exhibiting high resistance might be more effectively utilized. If the factors responsible for durability within a species were known, that timber exhibiting a preponderance of these factors would be more acceptable under conditions favoring decay. Products made from such timber could be expected to last longer in service than those manufactured from unselected material. There would also be the possibility of breeding highly durable genetic types within species exhibiting decay resistance. This might be accomplished by using as a seed source only those trees displaying the highest degree of durability factors.

Douglas fir (Pseudotsuga menziesii (Mirb.) Franco) is the predominant commercial timber species of British Columbia, accounting for 39 per cent of the total cubic volume harvested in 1953 (11). Of all lumber manufactured in the

Province in 1952, 47 per cent was Douglas fir (26). Virtually all of the softwood plywood produced on the Pacific Coast is made from this single species. Douglas fir is also widely utilized for poles and piling, and as a raw material for sulphate and mechanical pulping. Since the quantity of accessible timber in merchantable sizes is being continually reduced, it is expedient that our existing supply of this valuable species be extended as far as possible.

The useful life of timber under conditions favoring decay has been greatly extended through the use of wood preservatives. These preservatives give maximum protection to wood when they are applied by pressure methods. The heartwood of Douglas fir grown on the Coast is only moderately difficult to impregnate, but the Rocky Mountain form of this species is very difficult to penetrate (30). Douglas fir grown in the interior of British Columbia is notorious in this respect. Since some forms of its heartwood cannot be properly treated when desired, Douglas fir is often used under conditions favoring rapid decay without first having received a preservative treatment. The only alternative in this instance is to select naturally durable timber for such uses. This cannot be done until the factors influencing the relatively high durability of Douglas fir are understood.

It is apparent that the existing supplies of Douglas fir could be extended if the incidence and extent of decay in trees and timbers of this species were reduced.

Wood decay in general may be defined as those chemical and physical changes resulting from the activity of wood-destroying fungi. The fungi capable of such destruction require certain conditions in order to develop to their maximum level. One factor of major importance in their reaching this level is a suitable source of food. Wood substance is capable of supplying the necessary food requirements. Wood decay occurs as these fungi secrete specific enzymes capable of hydrolyzing the wood substance to forms that can be absorbed by the fungi. Separate enzymes act further upon these hydrolyzed products during the process of fungal metabolism.

Finholt, et al (16), have suggested two mechanisms by which introduced preservatives might prevent decay in wood. Their first hypothesis proposes that these substances actually interfere with the metabolic processes of the fungus. That is, the oxidation-reduction enzymes (endoenzymes) that normally react to digest the water-soluble compounds are upset in such a way that they are unable to function properly. Their second theory suggests that the enzymes secreted by the fungus (exoenzymes) are denatured so that they become incapable of hydrolyzing the wood complex. Their experimental evidence favors the second hypothesis. A high-boiling creosote was found to be non-toxic when mixed with malt agar, but highly toxic when used as a preservative in small wood blocks. Evidently, the hydrolytic enzymes were denatured in both cases,

but this action had no effect in malt agar, since the food source (maltose) is already in a form that allows its assimilation by the fungus. On the other hand, it is well known that fungi can be inhibited and killed in malt agar containing even a small concentration of highly toxic chemicals. In view of this fact, the first theory seems most appropriate. It appears that both the endo- and exoenzymes associated with a fungus may be disturbed by the presence of certain chemical compounds.

Different species of wood exhibit a widely varying ability to resist the action of wood-decaying organisms. Moreover, some variation in this ability exists from tree to tree within a single species. Attempts have been made to explain this variation in decay resistance by considering the general physical and chemical nature of wood. Zabel (57) concluded from a literature survey that no overall relationship exists between specific gravity and decay resistance within a species. When considering extremes within a species, however, very dense wood may be more durable than the lightest wood, since gaseous diffusion is at a minimum in the heaviest wood with the smallest void volume. Between different species, there is no correlation between density and durability. Some factor other than density is therefore largely responsible for the natural decay resistance exhibited by some woods.

Hawley, et al, (20) were the first to note a relation between the durability and chemical composition of wood.

When water extracts from a number of durable species were mixed with malt agar solutions, they were found to exert a toxic effect on fungal growth. The hot water extract of the heartwood was observed to be the most toxic, while similar extracts from the sapwood displayed very little fungicidal activity. Several other investigators (38, 39, 40, 49, 57) have since concluded that the decay resistance of durable wood species can be best explained by considering the nature of their heartwood extractives. Sapwood of all species is considered non-durable, except when it exists in the living tree. In this case, it is usually more resistant to decay than heartwood, since the higher moisture content of sapwood may present an unfavorable water-oxygen balance to most fungi. The inability of most wood-destroying fungi to function as true parasites also prevents sapwood decay *in vivo*.

Sherrard and Kurth (47) showed that the durability of redwood (*Sequoia sempervirens* (Lamb.) Endl.) was largely due to its hot-water extract, and that this varied with position in the stem. Southam and Erlich (49) and Roff and Atkinson (43) have concluded that various extractive portions of western red cedar (*Thuja plicata* D. Don) are toxic to fungi. Zabel (57) suggested that the water-soluble tannins of white oak (*Quercus alba* L.) are largely responsible for its decay resistance. Rennerfelt (38, 39, 40) investigated the effect of phenolic extractives in Scots pine (*Pinus silvestris* L.). He found that pinosylvin, and to a lesser extent, pinosylvin

monomethylether were toxic to fungi.

In summary, woods naturally unfavorable to fungi as a source of food are said to be durable, or to have a high degree of decay resistance. Species that exhibit this property have natural durability factors that actually inhibit fungal development. These are usually attributable to heartwood extracts. The chemical nature of heartwood extractives varies between species; those extracts showing the highest degree of toxicity are obtained from woods proven to be more durable in service. The chemicals in the heartwood extract probably exert their toxic effect by interfering with the highly specialized and imperfectly understood enzyme system of the fungus.

The durability of Douglas fir is intermediate between the very resistant and only moderately durable groups. Redwood and western red cedar are examples falling within the former classification, whereas the latter group is represented by spruce and hemlock (51). A literature survey revealed that no explanation had yet been given for the comparatively high natural durability of Douglas fir. Only one extractive component of Douglas fir, taxifolin, had been previously investigated for fungicidal activity. It was claimed to be a very weak fungicide (54). The statement was not supported by the correct bibliographical notation. This misunderstanding was rectified through correspondence with the author (12), who referred to unpublished results of Rennerfelt.

Further correspondence (37) revealed that only a cursory investigation had been made with taxifolin in malt agar. Pullularia, a genus primarily responsible for coniferous foliage diseases, was used as the test fungus. No numerical or comparative expression for toxicity was obtained, since the amounts of taxifolin added to the malt agar solutions were not determined.

It was the specific purpose of this work to investigate chemical extracts of Douglas fir heartwood in an attempt to ascribe the reason for its relatively high durability to certain extractive materials. In order to do this, it was necessary to first prepare various Douglas fir heartwood extracts, and then to divide these into less complex groups. Following this, an assessment (or bioassay) of each of these materials as fungicides was made.

II. CHEMICAL SEPARATION

Extraneous (or extractive) components have been defined as organic substances that may be extracted from wood by neutral solvents (23). There is no single universal solvent that will remove all of these various extractive components. In order to insure that the extractives have been quantitatively removed, a number of different solvents must be employed. Generally, separate extractions with alcohol or acetone, ether, and water are sufficient. Acetone leaches out the coloring matter, tannins and phlobaphenes. Ether has the property of removing oils, fats and resins. Cold water removes the soluble, short-chained carbohydrates, as well as some free acids and salts (28). Accordingly, these three solvents were used in this study to prepare the major extractive fractions.

Certain extraneous components of Douglas fir heartwood have been investigated by Schorger (46) and Pew (33,34). A systematic analysis of the entire extractive fraction has been made by Graham and Kurth (19). Their method was chosen because it provided a complete extraction procedure, with subsequent subdivision into known components. An outline of this extraction procedure is presented in Fig. 1.

SELECTION AND PREPARATION OF MATERIALS

A single codominant Douglas fir tree was felled on

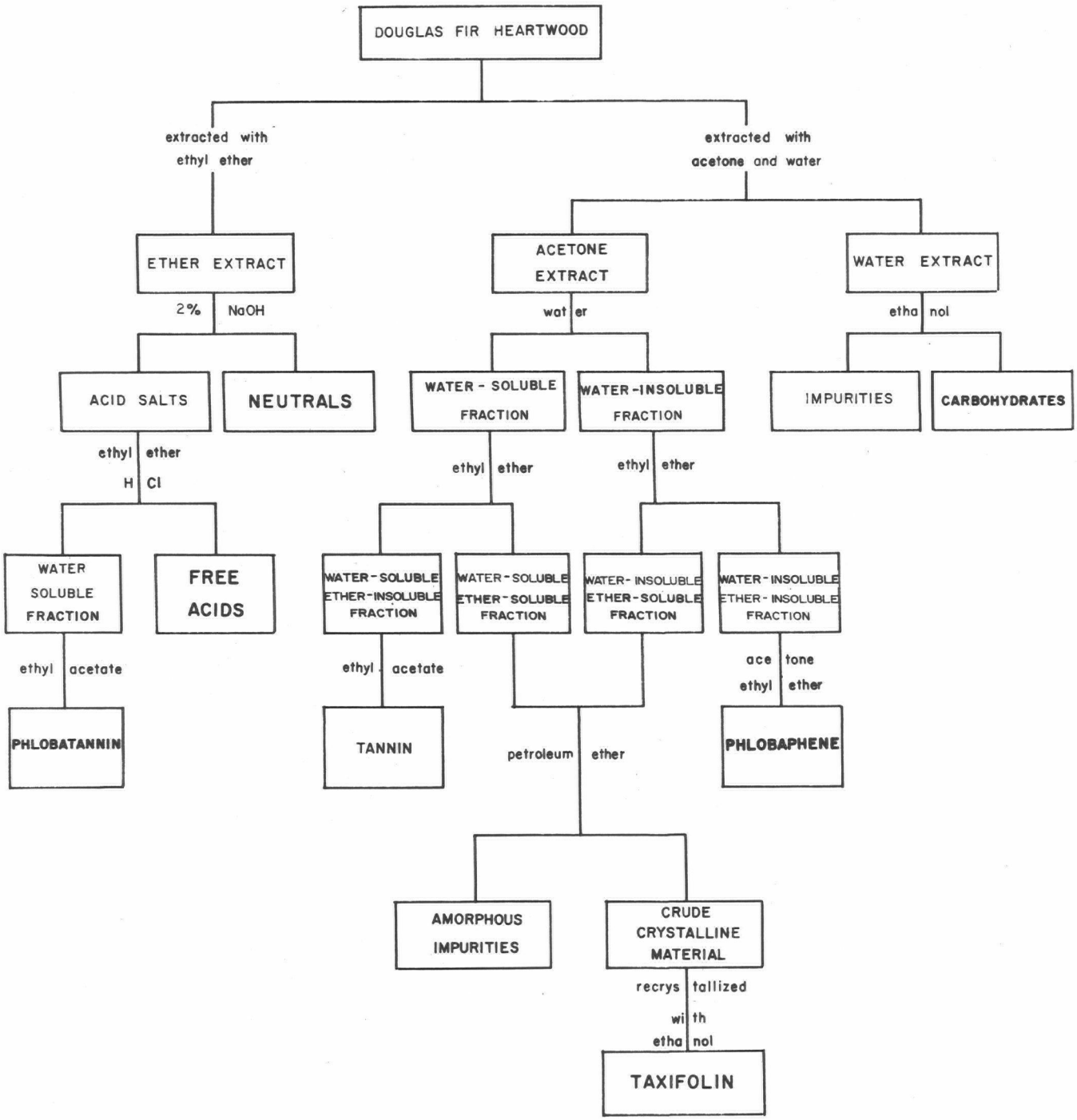


Fig. I. EXTRACTION PROCEDURE FOR ISOLATION OF EXTRANEOUS COMPOUNDS

October 1, 1954. This tree had been growing on a fir-hemlock-cedar site on the University forest at Haney, B.C. It measured 11.0 inches in diameter at breast height, and had a total height of 104 feet. Its age, including a correction for stump height, was approximately 83 years. A 1.5 inch band of apparent sapwood surrounded the heartwood.

Two four-foot bolts were selected from the butt end of the tree after felling. The butt end was preferentially chosen, since there is some evidence in certain other species to indicate that this portion is more durable than the top (4,47). A young, second-growth tree was selected, because work by Graham and Kurth (19) has indicated that heartwood material from this source contains a higher percentage of extractives than old-growth timber.

After slabbing the apparent sapwood from the first bolt, the heartwood portion was sawn into boards and subsequently reduced to planer shavings. These shavings were further reduced by grinding in a Wiley mill until they would pass through a 20-mesh screen. The resulting wood meal was then air seasoned until its moisture content dropped to approximately 15 per cent. An extended period of drying was avoided, since the volatile portion of the extraneous materials could be reduced by this procedure. It has been pointed out that extensive air seasoning decreases the amount of ether extract obtained from Douglas fir wood (19).

ISOLATION OF CHEMICAL COMPONENTS

1. Acetone extraction

A large glass tank was used to extract 1375 gms. of wood meal (oven-dry basis) with acetone. A fresh supply of solvent was introduced into the tank every two days, after first removing and storing the previous extract. Removal of the extract from the wood meal was expedited by using a coarse glass diffusion tube, to which suction was applied. The acetone was changed twice, so that a total of three extractive treatments was finally obtained.

The boiling point of acetone (56.5° C) is such that it can easily be distilled at low temperatures. Therefore, the extract was concentrated to a volume of 635 ml. by distilling the acetone. A 15 ml. aliquot from this concentrated extract was dried to constant weight under vacuum at 35° C. The total calculated yield of solid material was 2.31 per cent of the oven-dry weight of the wood.

A milky-white suspension was obtained upon addition of 250 ml. of water to the 620 ml. of concentrated extract. The solution was then further concentrated to a volume of 250 ml. under a vacuum at 45° C. The colloidal suspension was flocculated by adding a small quantity of sodium sulphate while constantly agitating the solution with a magnetic stirrer. After the white precipitate had settled, the clear supernatant solution was decanted and saved.

Both the clear liquid and the white precipitate were exhaustively extracted with ethyl ether. This was done to isolate that fraction of the total wood extract which is readily soluble in ether after initial extraction with acetone. It would not have been possible to quantitatively remove this material directly from the wood meal with ether (19,33,34). Four separate extracts were obtained by this procedure: a water-soluble, ether-insoluble fraction; a water-and ether-soluble fraction; a water-insoluble, ether-soluble fraction, and a water-and ether-insoluble fraction. To the two ethyl ether-soluble portions, petroleum ether was added to serve as a precipitating agent. After a sufficient quantity had been added to the constantly agitated solution, a limited amount of a red-brown, amorphous material was precipitated as impurities and discarded. A white precipitate followed after adding more petroleum ether. The resulting crude crystalline mass was recrystallized from ethanol four times. After the fourth recrystallization, the material was washed with water and dried under vacuum in an inert atmosphere of burner gas to prevent oxidation. Slightly over three grams of creamy-white crystals were produced, representing a yield of 0.23 per cent.

The water-soluble, ether-insoluble solution was exhaustively extracted with ethyl acetate to isolate the tannin. The solvent was removed under vacuum, but the amount of solids isolated was insignificant.

The water-and ether-insoluble fraction was redissolved in acetone and reprecipitated with ethyl ether several times in order to obtain a pure product. Finally, a brown, amorphous powder was isolated, dried and weighed. A yield of 0.014 per cent of the oven-dry wood was obtained.

2. Ether extraction

A large Soxhlet was employed to extract a fresh sample of wood meal with ethyl ether. A total of 2.75 kg. of wood meal (oven-dry weight) was extracted in consecutive 250 gm. portions for periods of 24 hours. Only enough ether was added between changes of wood meal to compensate for that which was lost through evaporation.

The resulting clear yellow extract was concentrated to 285 ml. by distilling the ether. A 10-ml. aliquot from this solution, dried to constant weight under vacuum, indicated that a total solids yield of 0.97 per cent of the oven-dry weight of the wood had been obtained. In view of this limited amount of material, it was inadvisable to resolve it into all its separate compounds. If this were done, there would probably have been too little of any single component to test for fungicidal properties. Instead, the extract was separated into three rather broad classifications for preliminary study. If any group proved to be highly toxic, a new extract could be prepared and further subdivided in an attempt to locate the exact source of

fungicidal activity.

The concentrated ether solution was extracted with a two per cent sodium hydroxide solution in a separatory funnel, thus forming salts of the free organic acids. The neutral material remained behind in an orange-colored ether layer. The ether solvent was evaporated at room temperature, so that as much of the volatile oil component as possible would remain behind. When no further loss of solvent was noted, the highly viscous material remaining was sealed and stored.

To the alkaline water solution, an equal volume of ethyl ether was added. The aqueous phase was acidified once again by the addition of HCl. Thus a water-insoluble acid fraction (ether layer) and a water-soluble fraction were obtained. After evaporating the ether under reduced pressure, a solid acid portion was obtained.

The water-soluble phase lost most of its color upon exhaustive extraction with ethyl acetate. A red-brown powder was isolated by evaporating the solvent in a vacuum oven. A yield of 0.07 per cent of the oven-dry weight of the wood meal was obtained.

3. Water extraction

After thoroughly air drying the acetone-extracted sawdust, it was leached with distilled water. The wood meal was extracted in a large glass jar at room temperature

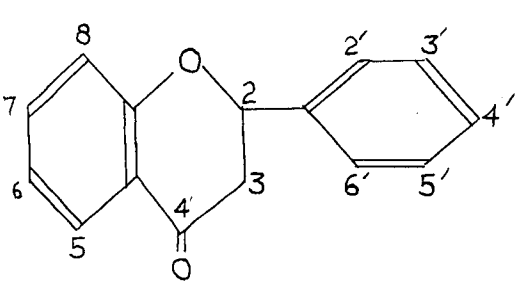
for a five-day period, with one change of water after two days. Extraction at elevated temperatures was avoided, as this could result in a gradual hydrolysis of the cell wall itself (50). The extract was concentrated in a constant temperature water bath at 80° C. A flocculent white precipitate was formed on the addition of four volumes of ethanol to the solution. The precipitate was collected by centrifuging, and then redissolved in water. Fractional reprecipitation with ethanol removed first the impurities, and a white precipitate resulted as more ethanol was added. The white precipitate was washed with ethanol and dried in the air. A yield of 0.36 per cent was obtained.

III. NATURE OF THE ISOLATED CHEMICAL COMPONENTS

ACETONE SOLUBLES

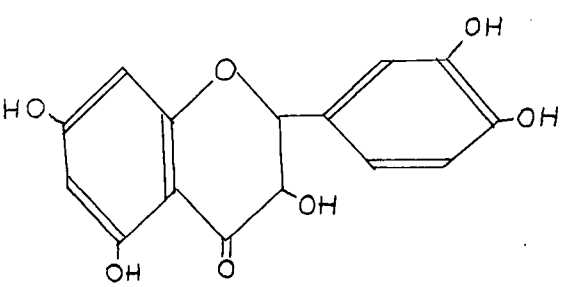
The major portion of the acetone extract was obtained in the form of creamy-white crystals, while the minor fraction was isolated as an amorphous powder.

The crystalline product obtained from the ether-soluble portion of the acetone extract was first described by Pew (33,34) and subsequently by Graham and Kurth (19). Its structure corresponds to that of a flavanone (I), specifically 3, 5, 7, 3/, 4/ - pentahydroxy flavanone (II) (33,34). This flavanone is readily oxidized to quercetin (5, 7, 3/ 4/ - tetrahydroxy flavanone) (III) (54). Since the former flavanone differs from quercetin only by two



I

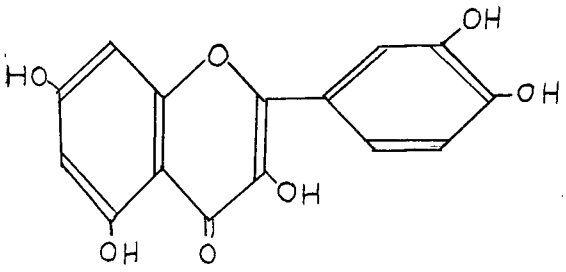
Flavanone Structure



II

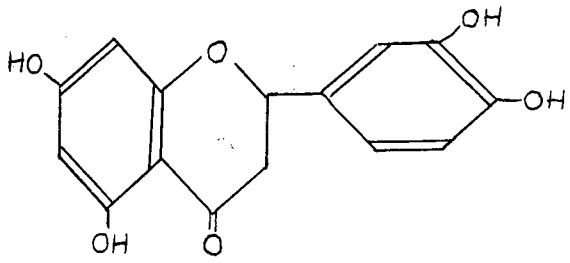
Taxifolin

(3,5,7,3/, 4/ - pentahydroxy flavanone)



III

Quercetin



IV

Eriodictyol

additional hydrogen atoms at the 2 and 3 positions with a consequent loss of a double bond, it is commonly called dihydroquercetin. Other equally common names are taxifolin and Douglas fir flavanone.

Eriodictyol (IV) is a reduction product of taxifolin, formed on addition of zinc dust and hydrochloric acid to an alcoholic solution of the flavanone. The development of a distinctive lavender color, reportedly characteristic for 3- hydroxy-flavanones, occurs in the course of this reaction (33,34). The deepness of the color produced can probably be relied upon to give a quantitative estimate of the amount of taxifolin present (3). Accordingly, a small amount of prepared crystalline product was weighed out and dissolved in methanol. The resulting solution was then analyzed quantitatively by the Wood Chemistry Section of the Vancouver Branch, Forest Products Laboratories of Canada. The consequent determination, which agreed well with the concentration that had previously been determined gravimetrically, indicated that the isolated

flavanone was relatively pure. Further evidence was obtained from a melting-point determination. The taxifolin crystals prepared from the acetone extract melted with decomposition at 233°-236° C. This compares favorably with the reported values of 240 - 242° C. (33,34) and 237° C. (19).

A colorimetric analysis of a methanol extract from the original mixed heartwood meal showed the total taxifolin concentration to be equal to 0.45 per cent. Thus the isolated yield of 0.23 per cent of crystalline product was slightly more than 50 per cent of the total. Pew (33,34) reported a yield (before recrystallization) of 0.62 per cent. Graham and Kurth (19) obtained a yield of 0.8 per cent.

The brown amorphous powder obtained from the water- and ether-insoluble fraction of the acetone extract has previously been determined as a phlobaphene (19). Phlobaphenes are defined as alcohol-soluble, water-insoluble condensation products of tannins (37). In wood analysis, native lignin is also removed by the same treatment, and probably is closely associated with the phlobaphenes (8,19). Only 0.62 per cent of the acetone extract was isolated as phlobaphene in this study, whereas 3.7 per cent had previously been reported (19).

This amorphous material was insoluble in alcohol, acetone and dioxane after isolation. There are two possible explanations for this insolubility: further condensation

between molecules may have occurred during purification, or the isolation procedure may have removed the associated material which normally exerts a peptizing effect (53). The phlobaphene was not investigated in the following toxicity studies, since the insolubility of this material prevented its proper impregnation into the substrates prepared for fungal attack.

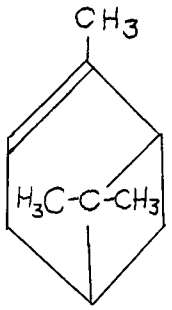
ETHER SOLUBLES

Three fractions were obtained from the ether extraction: A group of neutral materials, a free-acid portion, and a tannin-like substance. Each of these are discussed separately.

1. Neutrals

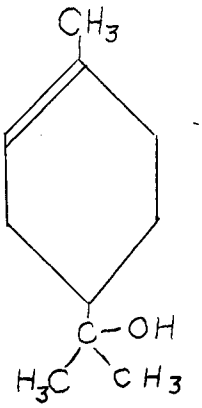
The neutral material remaining in the ether layer after extraction with dilute alkali probably contained a heterogeneous mixture of compounds. This product, which represented 10 per cent of the ether extract, probably included a certain amount of combined acids in addition to the unsaponifiable volatile oils and waxes (19). Its highly aromatic odor suggested the presence of the volatiles usually associated with Douglas fir oleoresin. Schorger (46) investigated this fraction and found that it contained a mixture of terpenes, predominantly 1 - α - pinene (V) and its derivative, 1 - α - terpineol (VI). Johnson and Cain

(24) reached a similar conclusion.



V

α-pinene



VI

α-terpineol

The remaining components in the neutral fraction have not been specifically identified, but probably consist of acids combined as esters, as well as high-carbon alcohols (sterols).

2. Free Acids

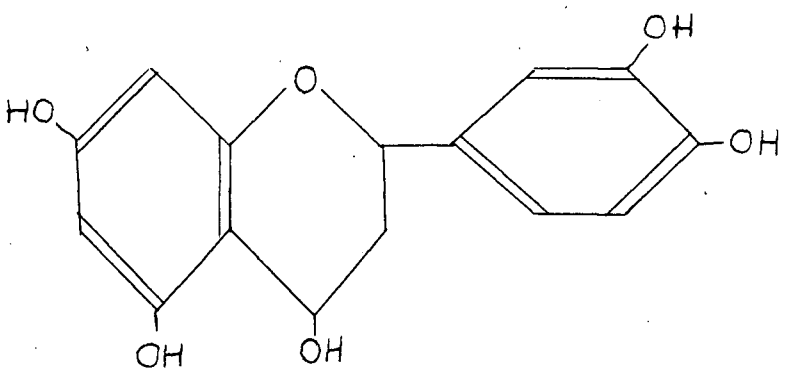
The free acid portion obtained by alkali extraction of the ether solubles was isolated as a dark-brown, tacky substance, having a consistency similar to taffy candy. This fraction has been reported to consist of an abietic-type resin acid in addition to several unidentified acids (19).

3. Phlobatannin

The red-brown amorphous powder, which was obtained by extracting the water phase with ethyl acetate, had the properties of a phlobatannin, since it produced a red-brown precipitate when heated with dilute mineral acid. The

precipitate from this reaction is termed phlobaphene, and thus phlobatannins are defined as phlobaphene-producing tannins (54).

Most naturally occurring phlobatannins will yield phloroglucinol and protocatechuic acid on alkaline fusion. These same compounds are produced when catechin (VII) is similarly treated (44). Catechin may be related to taxifolin inasmuch as it is thought to be an ultimate reduction product of quercetin. Attempts to convert taxifolin to a crystalline catechin have been unsuccessful, but the amorphous material obtained by these experiments has shown qualitative properties similar to catechin (33).



VII

Catechin

As pointed out previously, phlobaphenes may be synthesized by heating phlobatannins with dilute mineral acids. This conversion is accompanied by the loss of water (18,44). Thus phlobaphenes may be considered condensation products of phlobatannins. Similarly, phlobatannins

are believed to be condensed molecules, formed from the catechin nucleus (44). Moreover, catechin represents a reduced form of taxifolin. Therefore, dihydroquercetin may be a fundamental molecular type from which the more complex phlobatannin and phlobaphene arise. Such a relation has been suggested by Kurth, who termed pigments such as quercetin "precursors of the phlobatannins" (27). Russell has also indicated the possibility of this relationship (44).

WATER SOLUBLES

While drying in the air, parts of the white granular precipitate originally isolated from the water extract turned slightly brown in color. This may have been due to oxidation of gums and pectin-like substances that can occur in the water-soluble fraction along with the polysaccharides.

A small amount of the precipitated carbohydrate was hydrolyzed for one hour with sulphuric acid in a boiling water bath. After removing the acid by treating the solution with an ion-exchange resin, a paper chromatographic analysis was made. The chromatogram was developed for 12 hours in butanol-acetic acid-water in the volume ratio 4:1:5, and then sprayed with aniline trichloracetate (10). Both the brown and white portions of the carbohydrate fraction were found to consist of polymers of arabinose and galactose since only these sugars were detected after hydrolysis. A previous study of the hydrolyzate of the

water-soluble polysaccharide of Douglas fir has revealed the presence of galactose (90%), arabinose (9%) and xylose (1%) (50). Before this time, the principal carbohydrate had been regarded as galactan (19).

IV. BIOASSAY

The isolated components included the taxifolin, neutral, free acid, phlobatannin and carbohydrate fractions. These materials had to be evaluated for relative fungicidal activity. This was done by observing how a given chemical affected the growth of a fungal organism. In order that direct comparisons between the substances could be made, numerical expressions for their effectiveness were sought.

EXPERIMENTAL METHOD

A measure of the toxicity of a chemical may be obtained by introducing the material into a substrate, which is subsequently exposed to the action of a fungus for a specified length of time. Two general types of substrates have been employed to evaluate toxic effects on wood-destroying fungi. One type involves the use of some non-cellulosic medium such as malt agar or an aqueous nutrient solution to which the chemical may be added. In the second type, the chemical in question is added directly to the cellulose material such as wood, pulp or sawdust. One procedure involving each of these approaches is included in this study. These will be considered separately.

1. Non-cellulosic Medium

Non-cellulosic substrates for evaluating wood

preservatives have employed water or agar, to which nutrients have been added (2,15,20,38,39,40,43,47,49,57). The chemicals added as toxicants may not be present in the same form as they naturally occur in the wood; thus, the numerical results obtained might not necessarily apply to a cellulose-fungicide system. Nevertheless, the use of a non-cellulosic medium provides a satisfactory method of determining comparative toxicity of different substances (29).

If an aqueous nutrient solution is used, it is inoculated with a fungus which is then allowed to grow for a specified period of time. After the mycelium has been removed by filtering the nutrient solution, it is oven-dried and weighed. The amount of growth in solutions of varying chemical concentrations, as reflected by the weight of the mycelia, serves to indicate the degree of fungicidal toxicity. This method has one serious disadvantage: chemicals added in the form of water-insoluble solutions cannot be kept in a dispersed condition without the addition of an emulsifying agent. For this reason, this method was not seriously considered for this study.

When malt agar is employed as a substrate, radial measurements of mycelial development are taken periodically after inoculation with a fungus. The retarded rate of growth on malt agar containing various concentrations of chemicals can be used to express toxicity. This technique has been employed by several investigators (15,20,38,39,43,

47,49,57). The malt agar method is the most rapid one available for the evaluation of fungicides. Mycelial growth begins soon after inoculation so that data can be collected almost immediately, without waiting for a long incubation period. Its property of gelling quickly on cooling makes it possible to retain water-insoluble solutions in a dispersed state (29). Since two of the chemical fractions that were to be evaluated were insoluble in water-miscible solvents such as alcohol, malt agar was selected as the non-cellulosic medium.

Preparation of substrate--Difco malt agar was dissolved in distilled water and sterilized in 100-ml. quantities. In order to determine the effect of chemical concentration on toxicity, varying amounts of the prepared extractives were then added to the malt agar. Weighed amounts of taxifolin and phlobatannin were added in the form of alcoholic solutions, whereas the acid and neutral fractions were first dissolved in ether. The amount of chemicals added ranged between 0.01 gm. and 1.08 gm. One ml. of solution was the smallest amount added, while higher volumes (up to 4 ml.) were used to correspond to larger amounts of material. Preliminary tests had revealed that ether exerted only a slight toxic effect. Alchohol had previously been used to apply water-insoluble substances to malt agar (38,39). Malt agar solutions containing both alcohol and ether of various

concentrations were prepared for control purposes.

The concentration of material in the resulting malt agar solution was expressed in grams per cubic centimeter. This was converted to a percentage by multiplying by 100. Since the volume of malt agar used was always 100 cc., the weight of material added to the malt agar became a direct measure of the percentage concentration. The concentration of the chemical solvent in solution was also expressed as a percentage. Since between one and four ml. were added, the concentration in the malt-agar solution ranged from one to four per cent.

When the flasks containing the malt agar had cooled sufficiently after sterilization, the alcoholic chemical solutions were aseptically added and mixed with the malt agar by gently swirling the flasks. The material in the flasks was then poured into small Petri dishes (50 mm.). Eight dishes could be poured with each 100 ml. of prepared solution. The smaller Petri dishes were used in preference to larger ones, so that more replications within a single concentration would be obtained with the limited quantity of extractives on hand.

Essentially the same method was employed to prepare Petri dishes that would contain the ether-soluble groups. Since ether and water are immiscible, special precautions were necessary to insure proper dispersal of extractives in the ether solution. Ordinary swirling or stirring gives an unsatisfactory dispersion. The use of a Waring blender or

other high-speed mixer has been advocated for similar problems with oil-soluble wood preservatives (15). This modification divides the immiscible material into very small droplets so that a nearly homogenized solution is obtained. The ether-soluble chemical solutions were added to the malt agar and thoroughly mixed under aseptic conditions with a magnetic stirrer. Just before the solution gelled, it was poured into Petri dishes resting on an ice bath. The malt agar hardened within seconds, leaving the chemicals suspended as very finely dispersed droplets, discernable only with a handlens.

It was not anticipated that the water-soluble carbohydrate fraction would inhibit fungal growth. This was added to a plain agar solution in concentrations up to two per cent. The carbohydrate was sterilized separately from the agar solution to avoid hydrolysis of the polymers to simple sugars. After cooling, the agar was then added to the dry granular carbohydrate fraction, and the plates were poured in the usual manner. In testing the water-soluble fraction, the carbohydrate material replaced the malt which was used as a nutrient in the previous tests.

It was desirable to compare the results obtained using the wood extracts with a water-soluble chemical commonly employed as a wood preservative. Therefore, a parallel experiment, consisting of zinc chloride as the toxicant, was set up.

Selection of fungal organisms--A fungus must be able to grow reasonably well in culture to be of value in a malt-agar test. Secondly, the fungi selected to test extractives peculiar to a certain species should be commonly associated with this species. Finally, the test organisms must not be too sensitive to the alcohol and ether solvents used. A combination of these requirements led to the selection of Fomes annosus (Fr.) Cke. and Lentinus lepideus Fr. Initially, Poria incrassata (B.&C.) Curt. was chosen for use, but even low concentration of alcohol markedly retarded its growth. It was discarded in favor of Fomes annosus.

F. annosus is common in America, Europe, India and Australia, where it causes a butt-rot of conifers. It is the most important cause of heartrot in conifers grown in England, where many plantations of Douglas fir have been established (9). It usually enters the heartwood through dead roots, and eventually reduces the infested roots and butt to a series of elliptical white pockets. The fungus is also prevalent on damp mine timbers in both Europe and the United States (6,9). Douglas fir is the most common source of mine timbers in the Western States, and is being used increasingly for the same purpose in the East (32). F. annosus is used extensively in laboratory tests with malt agar, since it grows vigorously and is generally resistant to foreign chemicals.

Lentinus lepideus also has a world-wide distribution.

It is rarely found attacking a living tree, but is a very common factor in deteriorating timbers in service. It causes a typical brown rot, eventually reducing the wood to a series of brown cubes. In the United States, it is one of the most serious destroyers of coniferous railroad ties and poles (6). Douglas fir is utilized extensively for both of these purposes, especially in the Northwest (32), and is subject to the action of this fungus. In England it is the most important wood-destroying fungus attacking poles, paving blocks and mine timbers (9). L. lepideus grows at a moderately fast rate in malt agar, and is commonly used when wood preservatives are to be evaluated.

Inoculation of Petri dishes--Pure cultures of Fomes annosus and Lentinus lepideus were obtained from the Wood Pathology Unit of the Vancouver Branch, Forest Products Laboratories of Canada. From these original cultures, a number of transplants were made to plain malt agar. After an 18-day period (± 3 days), a piece of inoculum was taken from the actively growing margin and transferred to the outer edge of each Petri dish. The inocula were all cut with a number one cork borer (4 mm. diam.) to insure a uniform size. All inoculations were performed no later than 24 hours after preparation of the Petri dishes.

Incubation and measurement of growth--The temperature to which a fungal organism is exposed greatly influences its rate of growth. The optimum temperature for Fomes annosus

is 23° C, while 27° C is most suitable for Lentinus lepideus (9). Consequently, all the inoculated Petri dishes were incubated in a constant temperature chamber at 25° C. The plates were ordinarily not sealed because of the short duration of the experiment. However in the case of the dishes containing the volatile neutral fraction, masking tape was used to seal the dishes. This effectively prevented the loss of possibly toxic, volatile materials to the surrounding chamber atmosphere.

Readings were taken at approximately two-day intervals until the plates were covered with mycelium, or until the agar started to dry and crack through desiccation. Fomes annosus control plates were covered in six days, while Lentinus lepideus controls took a total of ten days.

The amount of radial growth between the center of the inoculum and the margin of the mycelium was measured in millimeters. Under comparable conditions, the replicates seldom varied more than four mm.

Experimental design--As has been previously stated, eight plates were poured for each concentration of a given chemical. Therefore, a total of four replications were available for testing the reactions of each fungus. Owing to the varying amounts of chemicals available, the concentrations examined were not standardized throughout the experiment. The concentrations tested for each given chemical may be read from the curves in Appendix A.

Malt-agar plates containing one, two and four per cent ether and alcohol were poured for controls. These solvent volumes corresponded to those actually used to dissolve the taxifolin, phlobatannin, neutral, and acid fractions. Petri dishes containing only malt agar were also inoculated to determine to what degree the ether and alcohol solvents themselves were toxic.

2. Cellulose Medium

Natural conditions of wood decay can be more closely approximated by exposing wood blocks to decay organisms under controlled conditions. The British standard for testing wood preservatives consists of exposing a treated wood block to the action of a fungus growing on malt agar in a Kolle flask (9). British and American workers now favor the soil-jar method of Leutritz (29), where a treated block is placed on top of a similar untreated block that has previously been buried in soil inoculated with a wood-destroying fungus. In both cases, the degree of decay is determined by the weight loss suffered by the wood block after an incubation period of three to nine months. The American method was adopted for this experiment, since it requires less manipulation, and more closely represents actual field conditions.

Preparation of soil jars--Preserving jars (16 oz.) were

half filled with a high-organic content soil that had previously been moistened to 45 per cent moisture content. A 1.5-x 2.5-x 5.0-cm. feeder block of Douglas fir sapwood, cut so that its long axis was parallel to the grain, was buried in the soil so that only its upper surface was exposed. The jars were then sterilized for one hour at 15 pounds steam pressure prior to inoculation.

Selection of fungal organisms--In a test of this nature, it is desirable to use fungi that rapidly decay timber in service, thus causing a significant loss in weight over a short period of time. Lentinus lepideus has been found to cause severe weight losses within four months, but for the same period of time, Fomes annosus decomposed wood blocks only five to seven per cent (9). Therefore, a substitute for F. annosus seemed advisable for the wood-block test.

Poria incrassata a common cause of dry rot, was selected for this purpose. This fungus is extremely active along the Pacific Coast and in the Southeastern United States, where the prevailing climate presents an agreeable situation for the decay of coniferous building timbers (6).

Inoculation and incubation of soil jars--The inocula used were cut from the margin of the fungus growing in malt agar, and pressed down into the soil directly adjacent to the buried feeder block. The jars were then incubated in diffuse light at room temperature for 30 days. At the end of this

time, the mycelia had become well-established in the soil and on the feeder blocks in most of the jars. An excess of jars had been prepared so that only those providing an even, well-developed mycelium were continued in the test.

Preparation of test specimens--If the object of this experiment had been the evaluation of a wood preservative, blocks of wood would have been impregnated with the preservative and then introduced into the previously inoculated soil jars. The toxicity of individual extractives cannot be tested in this way, since they are already present as a group in the wood samples. The toxic effect of an extractive can be determined indirectly by leaching it from wood blocks before they are exposed to a fungus. If the material removed is toxic, the extracted blocks can be expected to decay at a faster rate. The amount of decay that occurs in these extracted blocks will be reflected by their weight losses. The greater weight losses suffered by the extracted blocks as compared to the control samples serve to indicate the importance of the extracted component as a natural wood preservative.

Small samples were prepared from which extractives could be easily leached. It was necessary that a major proportion of end grain be exposed in order to facilitate penetration both for extraction and decay. The $\frac{3}{4}$-inch cubes normally used in soil-jar tests would not be leached

satisfactorily, nor would they undergo decay as rapidly as smaller pieces with more exposed end grain. Findlay (14) has suggested that thin pieces under 6 cc. will give significant weight losses over a short period. This is explained by the increased rate of gaseous diffusion when surface-volume ratios are high.

A radially sawn board 5.0 cm. in thickness was taken from the original test bolt for the preparation of wood-block samples. The amount and toxicity of heartwood extractives may vary with radial position in the stem of a tree (4,9,57). Such variations can be nullified by making all heartwood test blocks a uniform distance from the sapwood. This was done by ripping a 2.5 cm. band of outer heartwood from the entire length of the radially sawn board. The resulting strip was dressed to 1.5-x 5.0-cm. This was reduced to small blocks by cross-cutting at 0.6 cm. intervals. Each sample block thus measured 0.6-x 1.5-x 4.0-cm., the smallest dimension being parallel to the longitudinal axis of the wood fibers.

Ten randomly selected samples were then leached in either acetone, ether, or water. This corresponded to the preceding initial chemical analysis. The test blocks to be leached in acetone or water were placed in glass jars at room temperature for six days, to simulate the original conditions of the acetone and water extractions. Aspiration in the respective solutions was necessary to insure complete

immersion of the blocks. The ether extraction was carried out in a Soxhlet until the collected solvent was slightly colored, indicating a quantitative removal of the extraneous materials. A one-week period was sufficient for this purpose.

In order to establish an initial equilibrium weight of the extracted samples, they were conditioned to a moisture content of 30 per cent in a constant temperature-humidity chamber. This was done in preference to oven-drying, which might reduce toxic volatile substances. Heating the blocks may also increase the resin content at the surface of the acetone-and water-extracted samples and so increase decay resistance (52).

Exposure of test blocks--When the samples reached equilibrium weight, they were each fitted with a straight pin, and flame-sterilized immediately before placing in the soil jars. The blocks remained in the jars in diffuse light for two months at room temperature. When this incubation period had elapsed, the blocks were removed, brushed free of excess mycelium and oven-dried. Oven-drying is necessary in this case since severely decayed samples will come to a higher equilibrium moisture content if reconditioned in the same manner as initially (31). After drying completely, the blocks were weighed to determine their loss in weight.

Experimental design--Ten blocks were used in each of the

acetone, ether and water extractions. Another group of ten unextracted samples was included for control purposes. Five blocks from each treatment were subjected to decay by P. incrassata, while the remaining half were put into soil jars previously inoculated with Lentinus lepideus.

ANALYTICAL METHOD

1. Non-cellulosic Medium

It has been well established that a steadily decreasing amount of growth is obtained as the concentration of fungal inhibitors in malt agar is increased (5). In order to obtain a direct comparison between the extraneous compounds tested, an estimate was needed of the chemical concentration necessary to completely inhibit growth. Such a concentration is called the Total Inhibition Point (T.I.P.), and has been defined by Schmitz (45) as the minimum concentration allowing no signs of growth either on the malt agar or the inoculum plug itself. This should not be confused with the killing point, which is that concentration necessary to kill a fungus. At the T.I.P. the fungus is not necessarily killed. Most fungi have the ability to remain dormant over long periods of time without visible signs of growth.

As the chemical concentration increases, the amount of mycelial growth decreases in a curvilinear manner that

can be represented by a parabolic or hyperbolic function. The curves obtained by plotting these points on ordinary co-ordinate paper could be extrapolated to zero growth to find the T.I.P. Such a procedure with curvilinear functions often causes inaccurate results. Bateman (5) found that a straight line could be obtained if the logarithm of the per cent retardation of growth was plotted against the logarithm of per cent concentration of fungicide. While this relationship did not hold consistently at lower concentrations, it did apply between the T.I.P. and a concentration approximately one-third of that value. This linear relation was found to be valid not only with Fomes annosus, but with green plants as well. The data collected in this study have been analyzed after the method of Bateman.

The data obtained from measurements of fungal growth were first averaged for each individual chemical concentration, time, and organism. The resulting figures, representing the means of four measurements, were then plotted on ordinary co-ordinate paper along with their respective controls. The ordinate represented radial growth in millimeters, whereas time in days was plotted on the abscissa. With few exceptions, the points were found to assume a nearly perfect straight-line relationship. Bateman had previously made the same observation (5). A diagramatic sketch of such a graph is shown in Fig. 2.

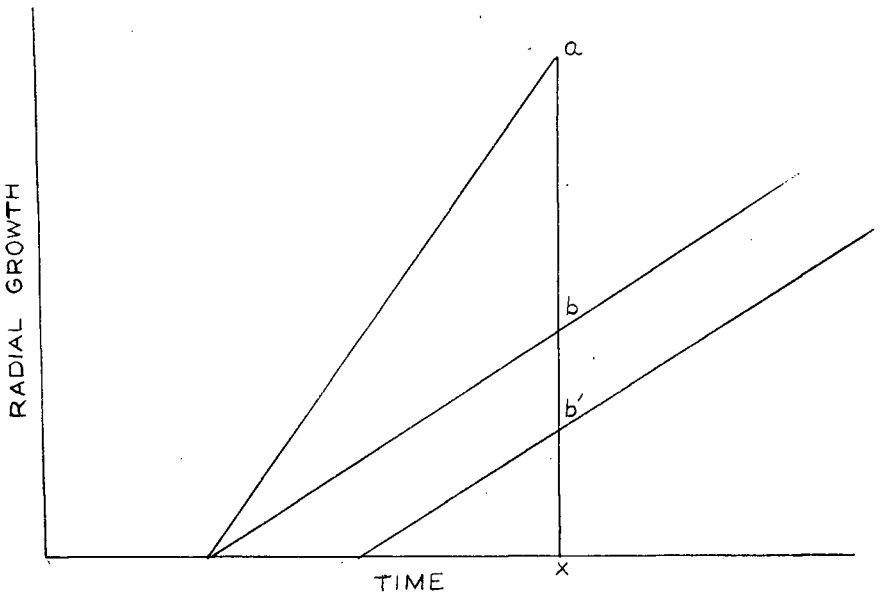


Figure 2. Techniques of Analysis

a - typical growth in control dishes
b - data of b/ corrected for rest period
b/ - typical growth in toxicant-containing dishes
ab - amount of retardation
$\frac{ab}{ax}$ - per cent retardation

Throughout the course of the experiments, growth rarely started immediately after transplanting the inoculum, even among the controls. A rest period of a day or more before growth commenced was not uncommon. A longer rest period was often required when an inoculum was planted in a Petri dish containing a fungal inhibitor. When growth finally started, however, the rate was sometimes nearly as fast as that of the controls. Bateman observed this same phenomenon, but added a correction factor for variable rest periods. This method was applied throughout the present work. If a chemical was found to induce a longer rest period than the control, the plotted points were first

connected in the usual manner to get the best possible linear fit. A line was then drawn parallel to this, through the point of intersection of the control line and the abscissa. Fig. 2 exemplifies this case, where line b/ represents the original data, b its correction for rest period, and a the control data.

Percentage retardation of growth was calculated in the following manner: the radial growth attained by the fungus in covering the control plate was considered as 100 per cent (line ax, Fig. 2). For the same time period, growth in a dish containing a fungicide reached only a fraction of the control (line bx, Fig. 2). Thus, the retarding effect could be represented by line ab, or the percentage retardation by ab/ax.

The figures obtained for per cent retardation were plotted against per cent concentration of chemical on double-log paper. The points were fitted to a straight line, and the line extrapolated to 100 per cent retardation. The concentration at which retardation of growth equaled 100 per cent represented the T.I.P.

2. Cellulosic Medium

The weight losses suffered by the test blocks while undergoing decay in the soil jars were expressed as a percentage on the basis of the original oven-dry weight of the specimens. Oven-dry values were calculated from the weights

obtained at an equilibrium moisture content of 30 per cent by dividing by 1.3.

The percentage loss in weight of the acetone-, ether- and water- extracted wood blocks was compared against the controls with Student's "t" test. The resulting values for "t" were evaluated at the five per cent confidence level. The lower weight losses of 20 per cent have a smaller variance about their mean than the higher values of 55 per cent. It was therefore necessary to first transform the original percentages into radians, thereby making the data independent of their respective means. The transformation arc sin $\sqrt{\text{percentage}}$ was used for this purpose (36).

RESULTS

1. Non-cellulosic medium

The odd-numbered curves in Appendix A show the growth of the test fungi in malt agar containing various concentrations of extractives in solution. Curves obtained with some very low concentrations are not shown, since they are co-incident with the control lines. Higher concentrations of toxic extractives obviously had an effect on the growth rate of both fungi. An example of this is presented in Fig. 3, where a series of Petri dishes containing successively higher concentrations of taxifolin were exposed to the action of F. annosus.

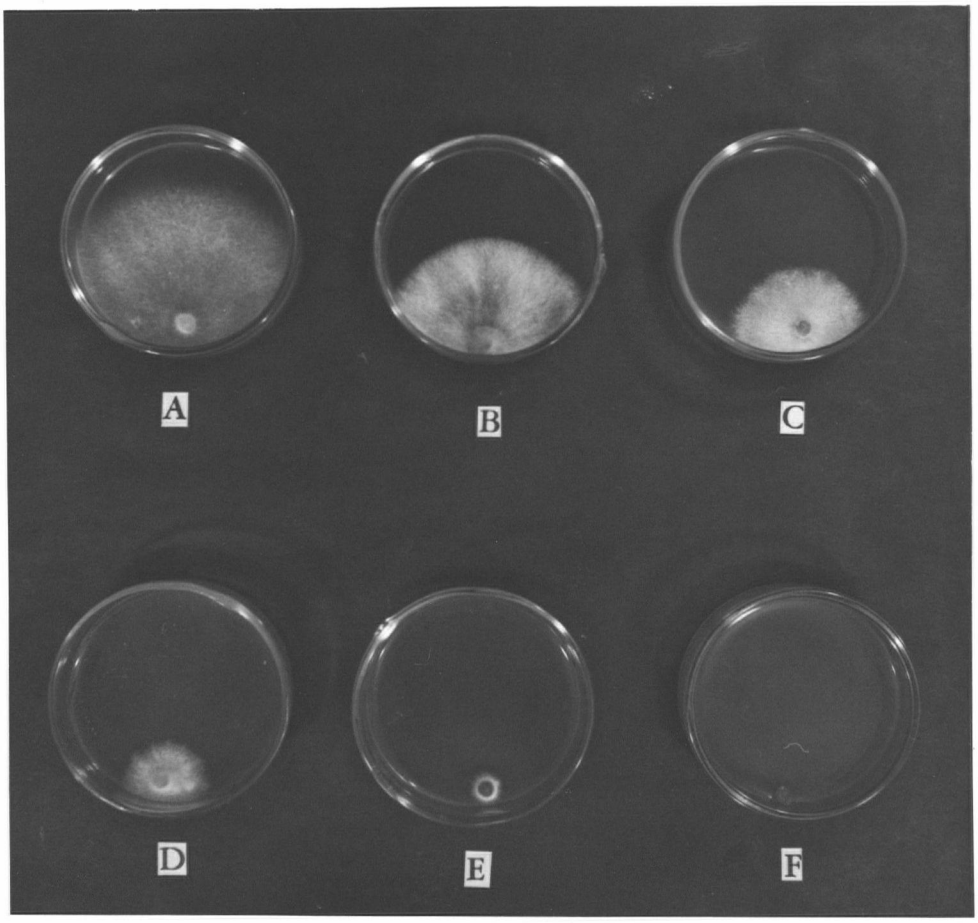


Figure 3

Effect of taxifolin concentration on growth of Fomes annosus in malt agar

A - control
B - 0.05%
C - 0.1%
D - 0.2%
E - 0.4%
F - 0.6%

Not only did the radial growth of the mycelium decrease with concentration, but the character of the fungal mat changed as well. Generally speaking, as concentration increased, the hyphae became more aerial in nature, the mycelium being particularly abundant around the inoculum. Mat margins also became more uniform, since the

usual advancing zone was lacking.

The even-numbered curves in Appendix A represent the logarithmic relation of per cent retardation plotted against per cent concentration. These have been extrapolated to 100 per cent retardation in order to determine the T.I.P. Values for T.I.P. are summarized in Table 1.

Table 1

Concentration of Chemicals in Malt Agar Needed to Completely Inhibit Fungal Growth at 25° C

Organism	Chemical or extractive tested	T.I.P.[1]
<u>Fomes annosus</u>	taxifolin	0.45
	phlobatannin	1.3
	neutrals	1.4
	free acids	1.1
	zinc chloride	0.16
<u>Lentinus lepideus</u>	taxifolin	0.70
	phlobatannin	1.6
	neutrals	4.7
	free acids	2.0
	zinc chloride	0.08

[1] Total inhibition point

Taxifolin was definitely the most toxic wood extractive investigated. However, none of the isolated groups approached the effectiveness of zinc chloride as a fungicide.

No concentration of ether or alcohol affected the growth of Lentinus lepideus in culture. However, Fomes annosus grew significantly slower in two per cent ether and four per cent alcohol. The curves obtained for these solvent concentrations were used as controls for those extractive groups requiring high amounts to properly dissolve.

2. Cellulosic Medium

The weight losses of the extracted wood blocks are presented in Appendix B. The averages obtained for each set of five samples are included in Table 2.

Table 2

Average Weight Losses of Extracted Wood Blocks in Soil Jars (2-month incubation)

Organism	Extractive treatment	Avg. wt. loss[1]
Poria incrassata	acetone	25.3
	ether	24.7
	water	29.7
	none	23.0
Lentinus lepideus	acetone	52.2*
	ether	51.8*
	water	44.7
	none	37.6

[1] Per cent of original oven-dry weight

*Significantly different from unextracted blocks (5% level)

The acetone- and ether- extracted samples exposed to the action of Lentinus lepideus suffered nearly equal and significantly higher weight losses than the controls. The results suggest that the three weakly toxic components investigated as sub-divisions of the ether extract may exert a combined fungicidal action equal to that of taxifolin. The water-extracted blocks, although not varying significantly from the controls, nevertheless show a somewhat higher weight loss. This was probably due to the removal of some water-soluble tannin substances along with the carbohydrate material.

The results obtained with Poria incrassata were not statistically significant when analyzed with the "t" test. A longer incubation period may be required with this fungus in order to obtain meaningful results, since the weight losses amounted to only half those of the samples infected with Lentinus lepideus.

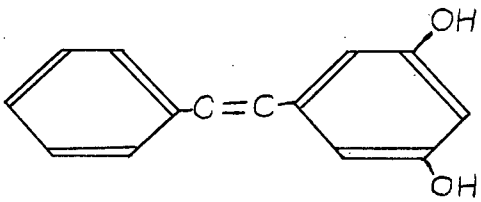
DISCUSSION

1. Taxifolin

Among the components tested in malt agar, only taxifolin was included in sufficient quantities to completely inhibit growth. No growth of either test organism occurred in dishes containing one per cent of this flavanone, and a concentration of as little as 0.6 per cent

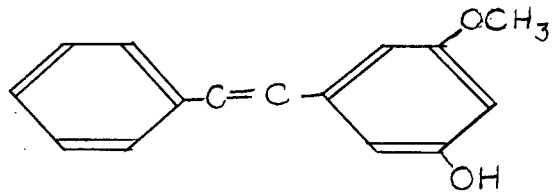
prevented L. lepideus from growing. After three weeks, the inocula from these three sets of dishes were transferred to fresh malt agar. Growth was not renewed, so it was concluded that these concentrations were sufficient to bring about death of the fungi. These points of killing concentration are included in the taxifolin growth curves (1 and 3) of Appendix A. They did not influence the extrapolation procedure, since these values represent a concentration above that of the T.I.P.

Rennerfelt (38,39) has determined the toxicity of pinosylvin (VIII) by the malt agar method. By plotting his data for Fomes annosus, a T.I.P. of 0.06 per cent is obtained. Pinosylvin was first isolated from Scots pine (Pinus sylvestris L.) and has since been found in a number of the hard pine (Diploxylon) group. Its monomethylether (IX) has also been isolated, not only from the hard pines,



VIII

Pinosylvin



IX

Pinosylvin monomethylether

but from the soft pine (Haploxylon) group as well (54). This monomethylether of Pinus acts as a weak fungicide, since concentrations slightly in excess of one per cent are required to completely inhibit growth of F. annosus (38,39).

In addition to both being phenolic in character, pinosylvin and taxifolin have certain other similarities. Both substances are soluble in ethyl ether only after they have been removed from wood with acetone or alcohol. Erdtman (54) has attributed this phenomenon to ether-insoluble "membrane substances" which envelop the phenolic molecules so that they are made inaccessible to ether. Extraction with alcohol or acetone apparently dissolves both the phenolic and ether-insoluble substances, allowing the taxifolin or pinosylvin to be subsequently soluble in ether.

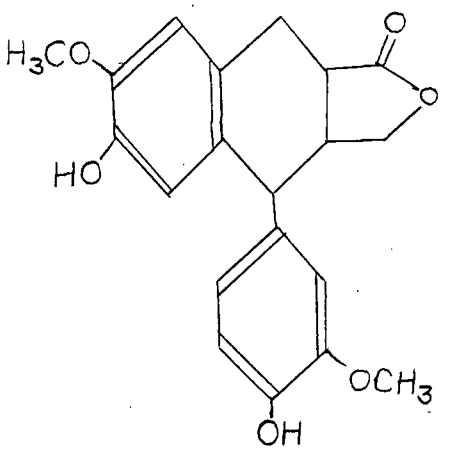
Therefore, there is some question as to whether taxifolin and similar phenolic compounds isolated by chemical means are identical with those occurring naturally in the wood. Their chemical nature and physical availability may differ when isolated. Further evidence to support this view arises when the insecticidal properties of taxifolin and pinosylvin are considered. Both compounds have been shown to be highly toxic to the West Indian dry-wood termite (Cryptotermes brevis Walker) (56). Wood blocks submerged in a 0.05 per cent taxifolin solution for ten minutes have remained free from attack for more than 42 months (55). The taxifolin present naturally in Douglas fir wood, however, apparently provides no protection whatsoever, since this species is readily attacked by the tropical dry-wood termite. The reason for this discrepancy may be one of availability. A wood block soaked in a taxifolin solution would present an

even distribution of pure compound to an attacking agency. The naturally occurring substance is probably distributed unevenly, and may be largely inaccessible to wood-destroying insects by existing in an intimate form with other materials.

Taxifolin has been reported to exist naturally in a average concentration of about one per cent in Douglas fir heartwood (33,34). The combined concentration of pinosylvin and its monomethylether in pine heartwood amounts to about 0.8 per cent of the dry weight of the wood (38,39). However, the less toxic pinosylvin monomethylether is present in amounts three to four times that of the parent pinosylvin (54). Thus, the combined fungicidal effect of these two pine substances should be expected to approach that of taxifolin in Douglas fir heartwood. Evidence to substantiate this view has been provided by Smith (48), who classes Douglas fir and Scots pine in the same natural durability group by service test experience.

Another related phenolic molecule present in the Pinaceae is conidendrin (X). This has been found in many species of the non-durable Tsuga, Picea and Abies genera. Like the particular phenols of the pines and Douglas fir, this compound cannot be directly extracted with ether. The non-toxic nature of this substance, as well as the decreased potency of pinosylvin monomethylether, may be explained by the substitution of methoxyl groups for hydroxy radicals.

Both pine and Douglas fir heartwoods are resistant



X

Conidendrin

to pulping by the normal sulphite method (21,35,54). It was formerly thought that the phenolic extractives reacted with lignin to form an insoluble phenolic lignin complex (35,54). Recent evidence has indicated that these phenolic compounds may inhibit pulping by decomposing the cooking liquor (21). When taxifolin is reacted with bisulphite, it is oxidized to quercetin. The reduction product of this reaction is probably thiosulphate, which in turn may catalyze further bisulphite decomposition to sulphuric acid (21). The acid thus formed can precipitate the calcium ions as a sulphate.

2. Phlobatannin

The phlobatannin fraction dissolved in alcohol with more difficulty than taxifolin. This was not surprising, inasmuch as the phlobatannin molecule is more complex than taxifolin, and may even be a condensation product of the flavanone. Solubility decreased with increasing molecular

weights among the taxifolin, phlobatannin and phlobaphene fractions. The differences in toxicity between taxifolin and phlobatannin indicated that fungicidal activity also decreased with increasing molecular weights. The most highly condensed molecule in this series, the phlobaphene complex, was not tested for toxicity. Its high degree of insolubility in all solvents suggested that it would have very limited fungicidal properties. This is further evidenced by the fact that native lignin is usually associated with the phlobaphene complex (8,19). A native lignin fraction has been isolated from most woods, including those having low natural durability. This implies that in all probability, native lignin does not function as a fungicide.

An independent investigation on a Douglas fir sample had revealed that the taxifolin concentration was four times greater in the outer heartwood than in the central pith area (25). The possible relationship between taxifolin and phlobatannin has been previously discussed. It was felt that the low concentration of taxifolin in the inner heartwood might have been the result of its being slowly transformed to phlobatannin over a period of years. The taxifolin produced in the outer heartwood might have been formed too recently to condense to any extent. Consequently, a rough quantitative test for phlobatannin was made to see if the above was, indeed the case.

Samples from the outer and inner heartwood were

extracted with ether, and the phlobatannin was isolated in ethyl acetate solution in the customary way. Douglas fir and redwood phlobatannin had previously been shown to absorb ultraviolet light strongly in a wave length of 280 mu (8,19). Spectophotometric analysis of the two phlobatannin extracts subsequently showed the ratio of tannin in the outer and inner heartwood to be 5:3. From these results, it would appear that taxifolin is not converted to tannin, if it is assumed that the amount of taxifolin originally produced is constant throughout the life of the tree. Unfortunately, there are no precise quantitative methods available for better tannin comparisons.

The foregoing observations are based on only one sample, and thus provide only an indication at best. It is still quite possible that taxifolin may condense to phlobatannin, but was not detected because of further condensation to phlobaphene. Fundamental work is needed in this field to definitely establish the distribution pattern of these three related substances.

3. Free Acids

Growth curves of Fomes annosus in malt agar with the acids included are presented in Fig. 13, Appendix A. The importance of correcting for rest period is forcibly illustrated here. Growth in a concentration of 0.8 per cent acid did not start until two days after growth began in the one

per cent dishes. Once started, however, its faster rate of growth produced a curve of higher slope. Each of these concentrations were corrected for rest period in the usual way. The curve representing the lower concentration was then found to fall above that of the higher. These corrected curves are illustrated by dotted lines in Fig. 13, Appendix A.

4. Neutrals

The concentration of the neutral ether fraction required to completely inhibit growth varied widely, depending on the organism involved. F. annosus is only moderately resistant, since the T.I.P. was determined as 1.4 per cent. Lentinus lepideus apparently is very resistant, requiring a calculated concentration of almost five per cent (Table 1.) This organism is also unusually resistant to oil-soluble preservatives such as creosote (9). Like the neutral fraction, creosote contains a heterogeneous mixture of water-insoluble, high molecular-weight hydrocarbons (42).

5. Water Solubles

The carbohydrate fraction apparently did neither support nor hinder fungal development in plain agar. Therefore, the water-soluble fraction from Douglas fir seems to function neither as a food source nor as a toxicant to fungi. Since the hydrolyzate was 90 per cent galactose, it can be concluded that the carbohydrate is largely a galactan (50).

Galactans, unlike mannans and pentosans are quite resistant to attack by wood-destroying fungi (7).

6. Zinc Chloride

The concentrations of zinc chloride necessary to inhibit growth agree well with data previously reported by Richards (41). L. lepideus is extremely sensitive to zinc chloride; a concentration of less than 0.1 per cent is sufficient to kill the fungus. The T.I.P. of 0.16 per cent attained with Fomes annosus represents the average sensivity of the majority of wood-destroying fungi. A taxifolin concentration of three times this latter amount is needed to prevent growth of F. annosus in malt agar. Even higher multiples of the other extraneous materials are required to inhibit fungal growth. The naturally occurring extractive materials, however, may eventually prove superior to zinc chloride in certain instances. Due to its water soluble properties, the salt leaches out of treated timbers under wet exposure conditions (22). The water-insoluble nature of the fungicidal Douglas fir extractives permits more permanent protection than is afforded by zinc chloride.

V. CONCLUSION

The specific concentrations of wood extractives needed to inhibit growth in malt agar should not be interpreted as representing the amount of material that must necessarily be present in wood to prevent fungal growth. Rather, they serve to indicate the relative importance that a component may play in protecting wood from decay. On the basis of the data collected in this study, it appears that taxifolin may be largely responsible for the moderate decay resistance of Douglas fir.

Since the discovery of the fungicidal properties of pinosylvin and its monomethylether, it has been suggested that trees containing high concentrations of these substances evenly distributed throughout the heartwood should be used for breeding purposes (13). The lumber produced from such strains would be inherently more durable. In order for this work to proceed, a simple colorimetric test has been developed for the semi-quantitative estimation of pinosylvin in the field (13). If a comparable test were available for the quantitative analysis of taxifolin, Douglas fir trees with high amounts of this flavanone could be selected for racial improvement. Work is now in progress to estimate taxifolin quantitatively from methanol extracts of Douglas fir (3). Nevertheless, a more rapid means of estimation in the field is still needed, since the above method is inapplicable to

wood *in toto*.

Western larch (*Larix occidentalis* Nutt.) has also been reported to contain taxifolin (17). Heartwood of this species, although somewhat easier to penetrate than Interior-type Douglas fir, still does not accept preservatives easily (30). Therefore, in both Interior Douglas fir and western larch, taxifolin will most often be the main deterrent to decay. Taxifolin concentration should be considered when lumber derived from these species is to be used under conditions where wood-destroying fungi might be active.

Chemical stimulation is being employed to increase resin production in the southern pines (32). A series of papers will soon be published on other methods of stimulating trees to produce greater amounts of extraneous materials (1). From such studies, economical methods may be developed to grow trees with greater proportions of fungicidal extractives.

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APPENDIX A

Growth and total inhibition point curves of *Fomes annosus* and *Lentinus lepideus* in malt agar containing various concentrations of extractives

. . .

Note: The linear relationship between log per cent retardation of growth and log per cent concentration holds only between the T.I.P. and one-third of that concentration. Hence points representing some lower concentrations were not considered in fitting the straight lines.

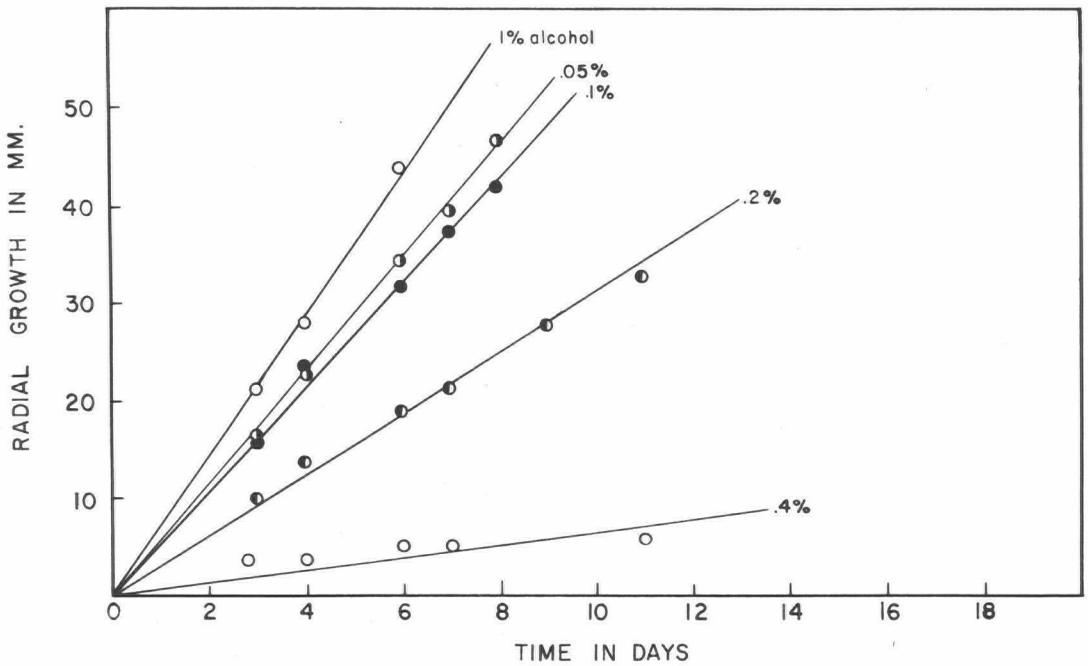
Figure 1.

Rate of growth of Fomes annosus on malt agar containing different concentrations of taxifolin.

(1% alcohol = control (0.00% taxifolin)

Figure 2.

Retardation of growth of Fomes annosus on malt agar containing different concentrations of taxifolin.


1% alcohol
.05%
.1%
.2%
.4%
RADIAL GROWTH IN MM.
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
TIME IN DAYS

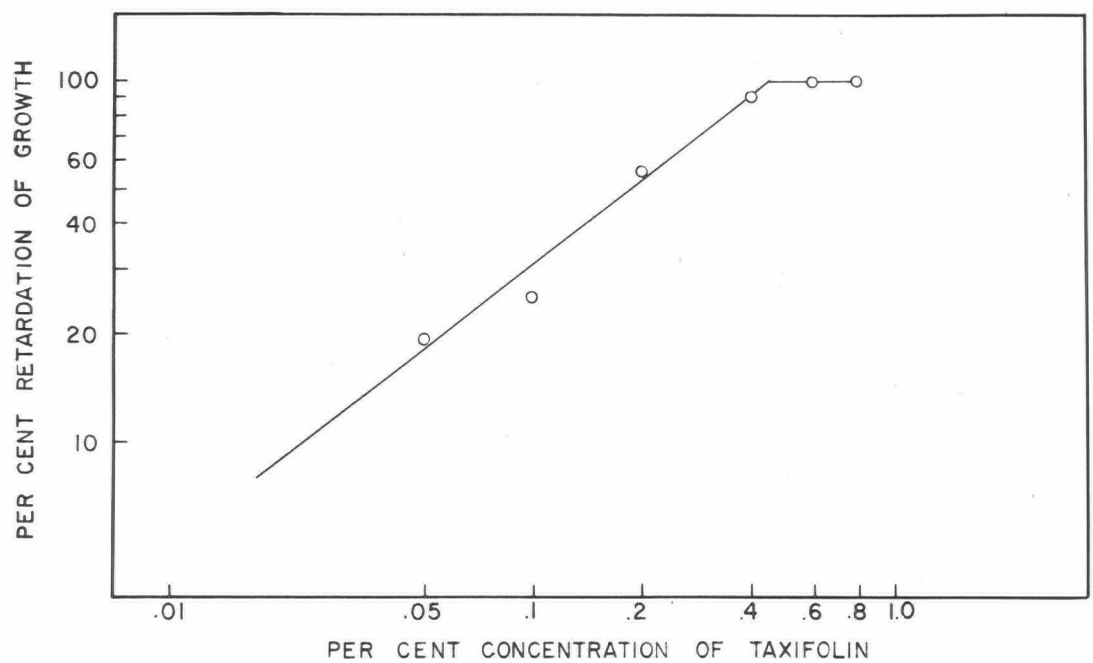

PER CENT RETARDATION OF GROWTH
100
60
40
20
10
.01
.05
.1
.2
.4
.6
.8
1.0
PER CENT CONCENTRATION OF TAXIFOLIN

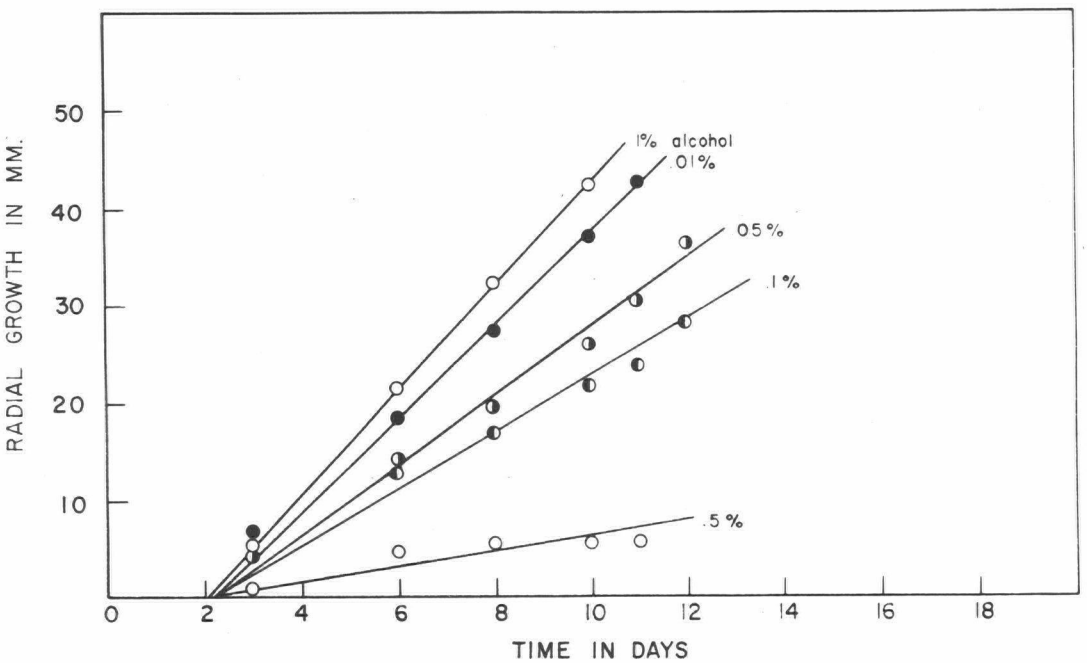
Figure 3.

Rate of growth of Lentinus lepideus on malt agar containing different concentrations of taxifolin.

1% alcohol = control (0.00% taxifolin)

Figure 4.

Retardation of growth of Lentinus lepideus on malt agar containing different concentrations of taxifolin.


RADIAL GROWTH IN MM.
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
TIME IN DAYS
1% alcohol
.01%
0.5%
.1%
.5%

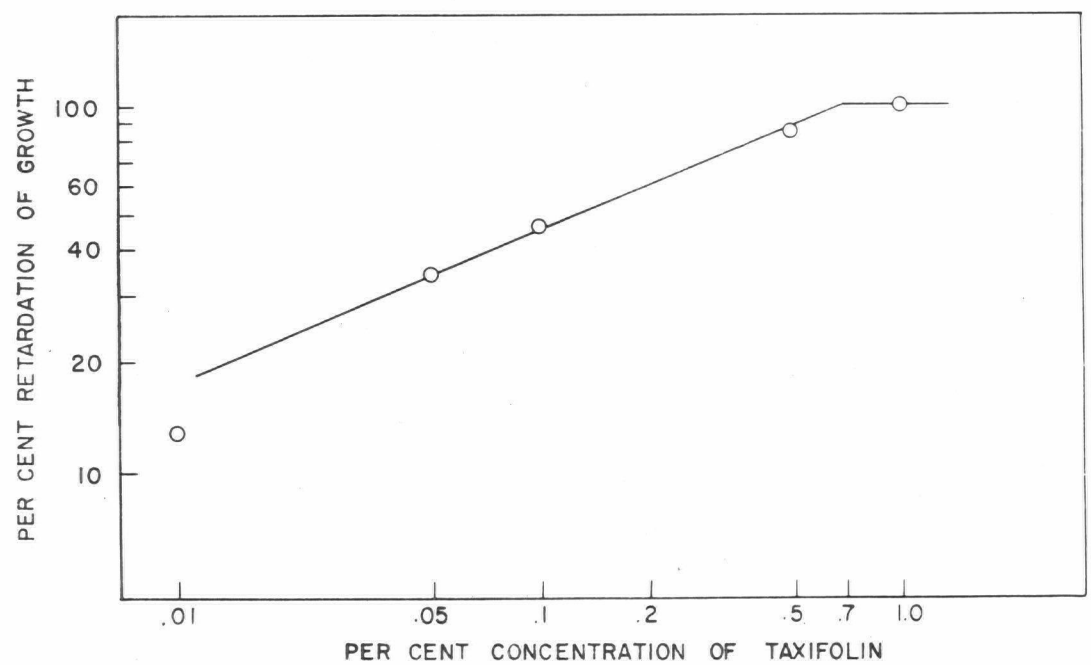

PER CENT RETARDATION OF GROWTH
100
60
40
20
10
.01
.05
.1
.2
.5
.7
1.0
PER CENT CONCENTRATION OF TAXIFOLIN

Figure 5.

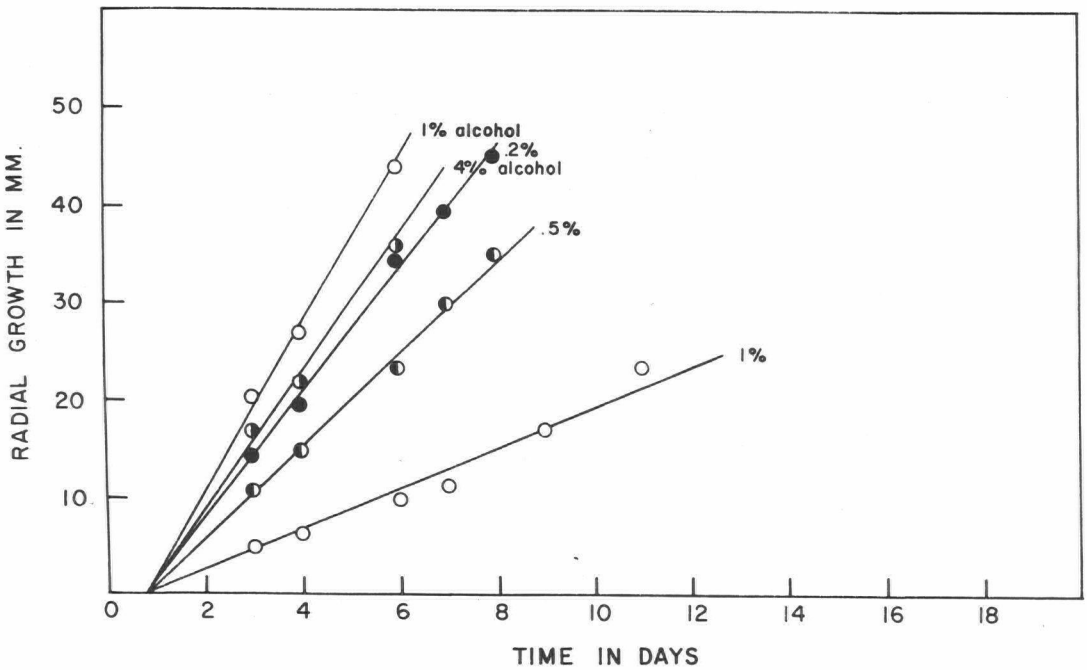
Rate of growth of Fomes annosus on malt agar containing different concentrations of phlobatannin.

1% alcohol = control for 0.2% and 0.5% phlobatannin

4% alcohol = control for 1% phlobatannin

Figure 6.

Retardation of growth of Fomes annosus on malt agar containing different concentrations of phlobatannin.


RADIAL GROWTH IN MM.
50
40
30
20
10
1% alcohol
.2%
4% alcohol
.5%
1%
0
2
4
6
8
10
12
14
16
18
TIME IN DAYS

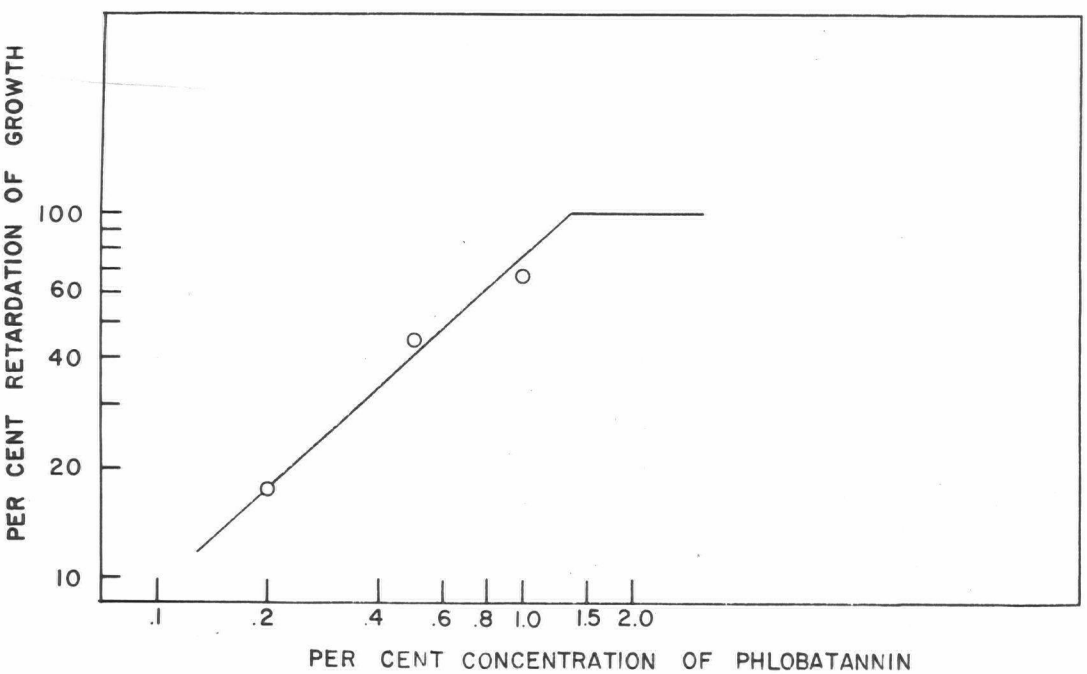

PER CENT RETARDATION OF GROWTH
100
60
40
20
10
.1
.2
.4
.6
.8
1.0
1.5
2.0
PER CENT CONCENTRATION OF PHLOBATANNIN

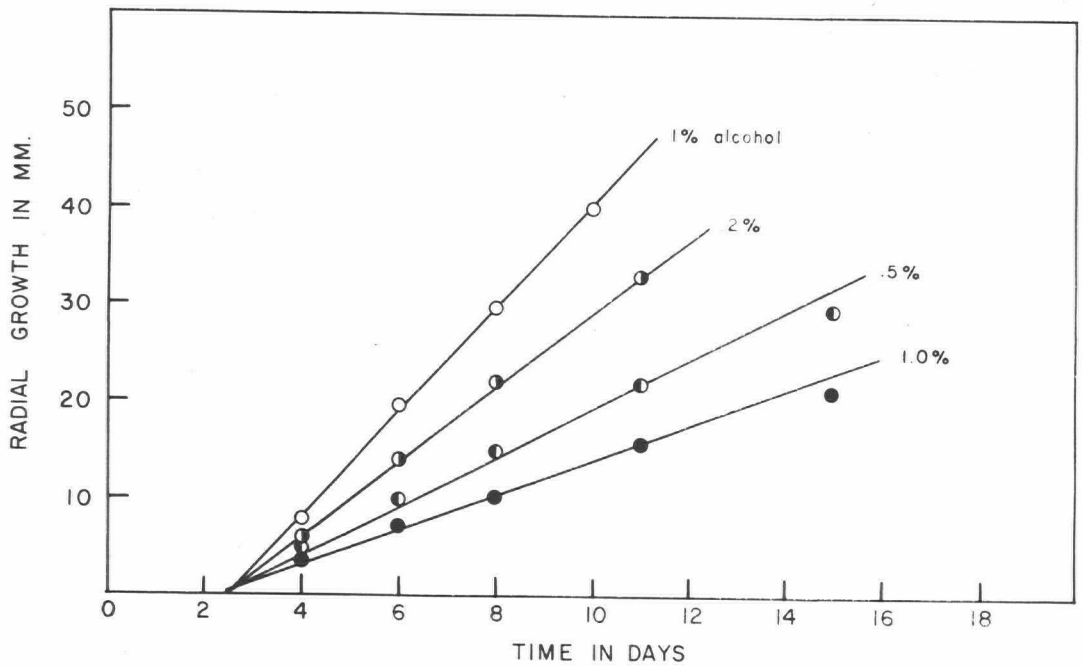
Figure 7.

Rate of growth of Lentinus lepideus on malt agar containing different concentrations of phlobatannin.

1% alcohol = control

Figure 8.

Retardation of growth of Lentinus lepideus on malt agar containing different concentrations of phlobatannin.


RADIAL GROWTH IN MM.
50
40
30
20
10
1% alcohol
2%
.5%
1.0%
0
2
4
6
8
10
12
14
16
18
TIME IN DAYS

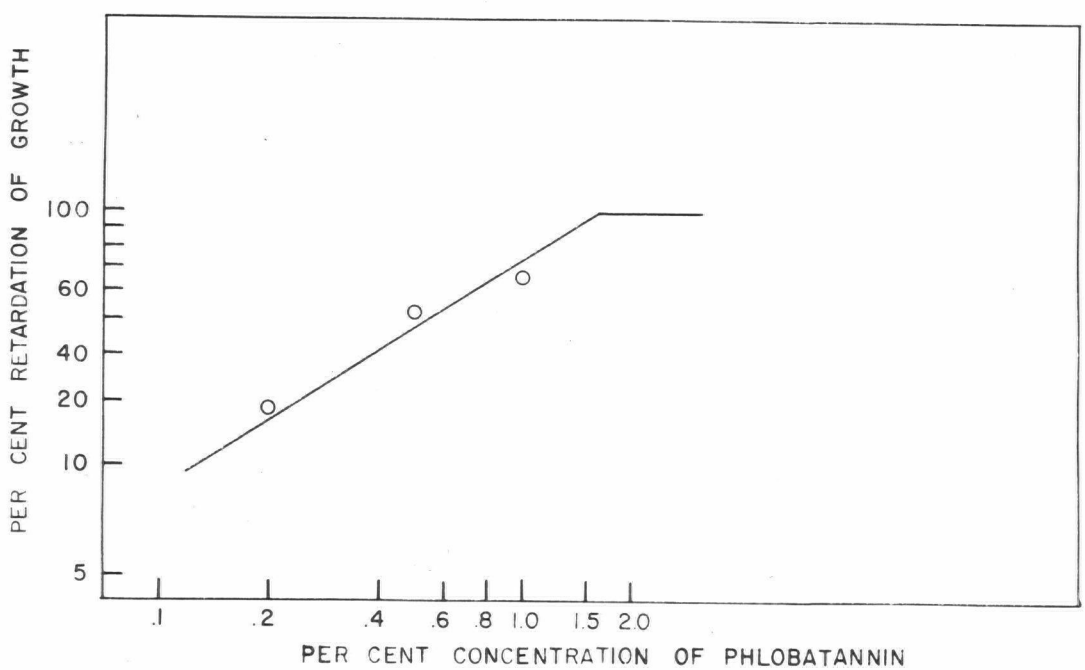

PER CENT RETARDATION OF GROWTH
100
60
40
20
10
5
.1
.2
.4
.6
.8
1.0
1.5
2.0
PER CENT CONCENTRATION OF PHLOBATANNIN

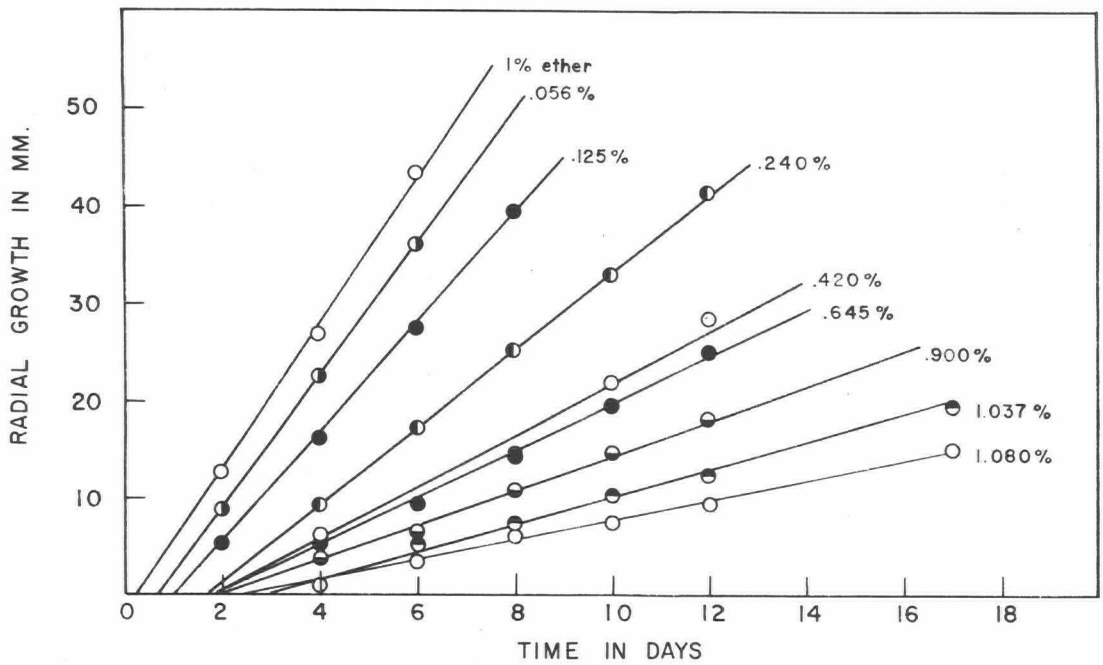
Figure 9.

Rate of growth of Fomes annosus on malt agar containing different concentrations of neutrals.

1% ether = control

Figure 10.

Retardation of growth of Fomes annosus on malt agar containing different concentrations of neutrals.


RADIAL GROWTH IN MM.
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
TIME IN DAYS
1% ether
.056 %
.125 %
.240 %
.420 %
.645 %
.900 %
1.037 %
1.080 %

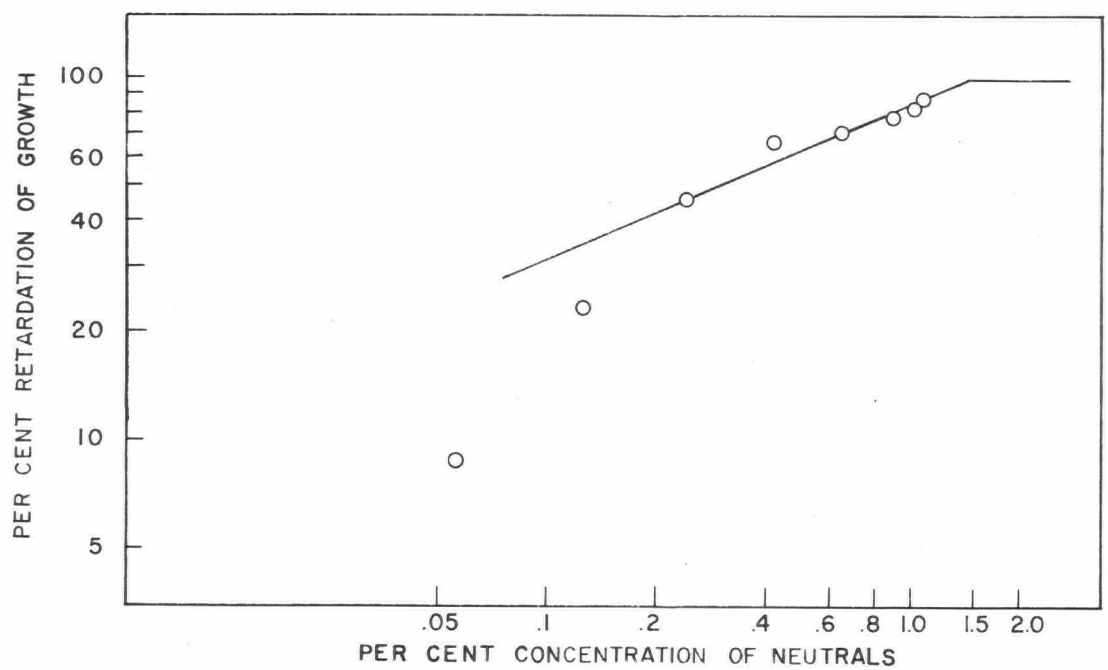

PER CENT RETARDATION OF GROWTH
100
60
40
20
10
5
.05
.1
.2
.4
.6
.8
1.0
1.5
2.0
PER CENT CONCENTRATION OF NEUTRALS

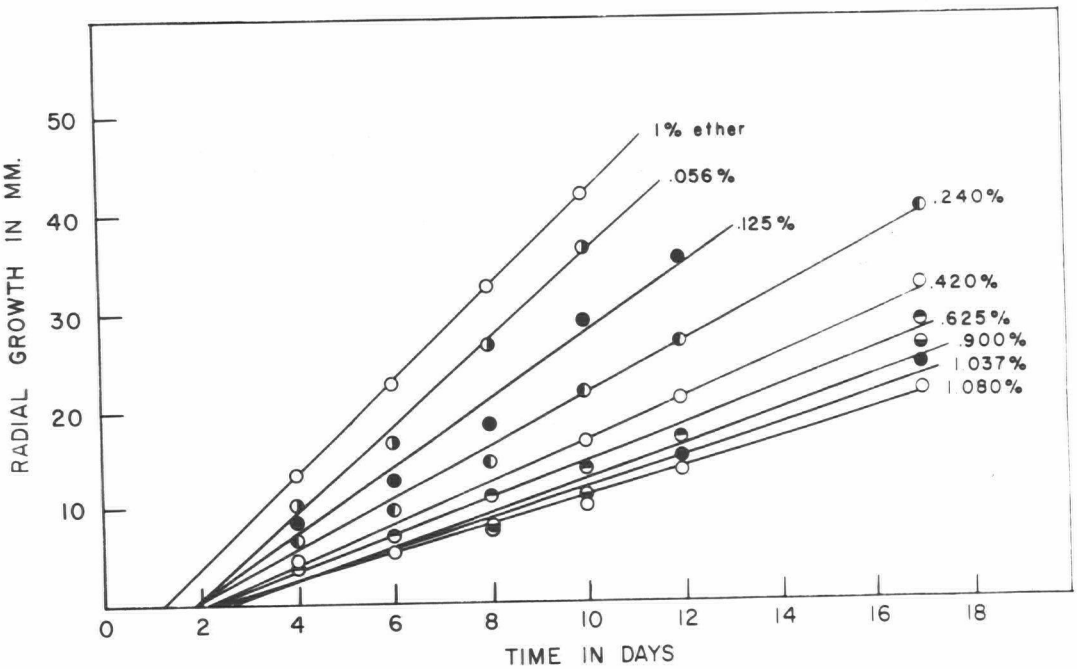
Figure 11.

Rate of growth of Lentinus lepideus on malt agar containing different concentrations of neutrals.

1% ether = control

Figure 12.

Retardation of growth of Lentinus lepideus on malt agar containing different concentrations of neutrals.


RADIAL GROWTH IN MM.
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
TIME IN DAYS
1% ether
.056%
.125%
.240%
.420%
.625%
.900%
1.037%
1.080%

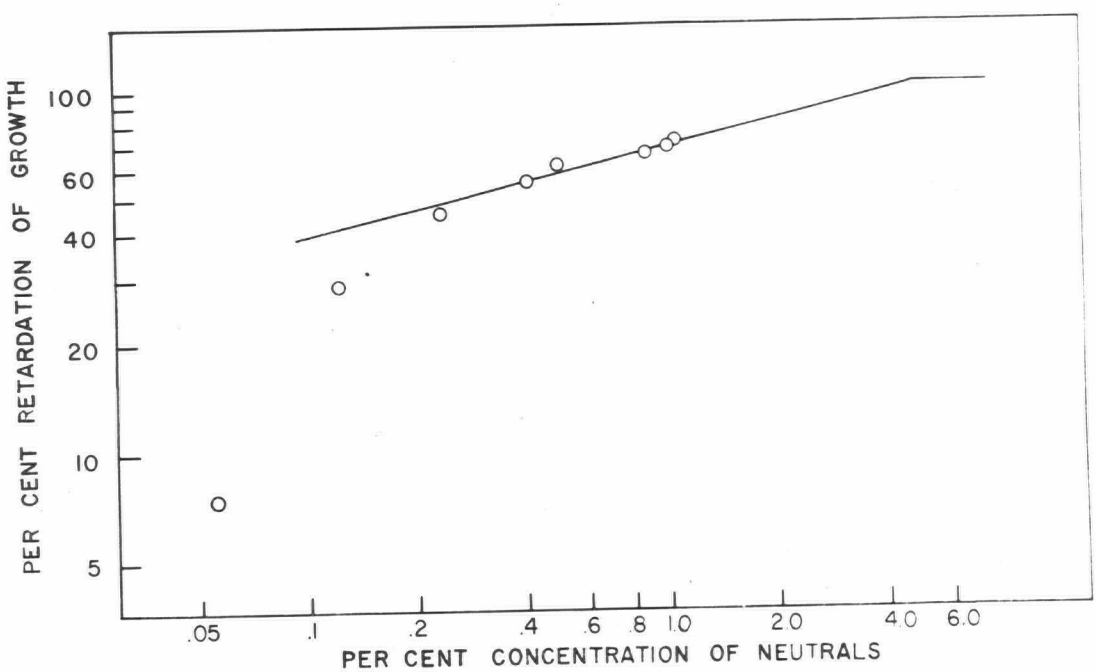

PER CENT RETARDATION OF GROWTH
100
60
40
20
10
5
.05
.1
.2
.4
.6
.8
1.0
2.0
4.0
6.0
PER CENT CONCENTRATION OF NEUTRALS

Figure 13.

Rate of growth of Fomes annosus on malt agar containing different concentrations of free acids.

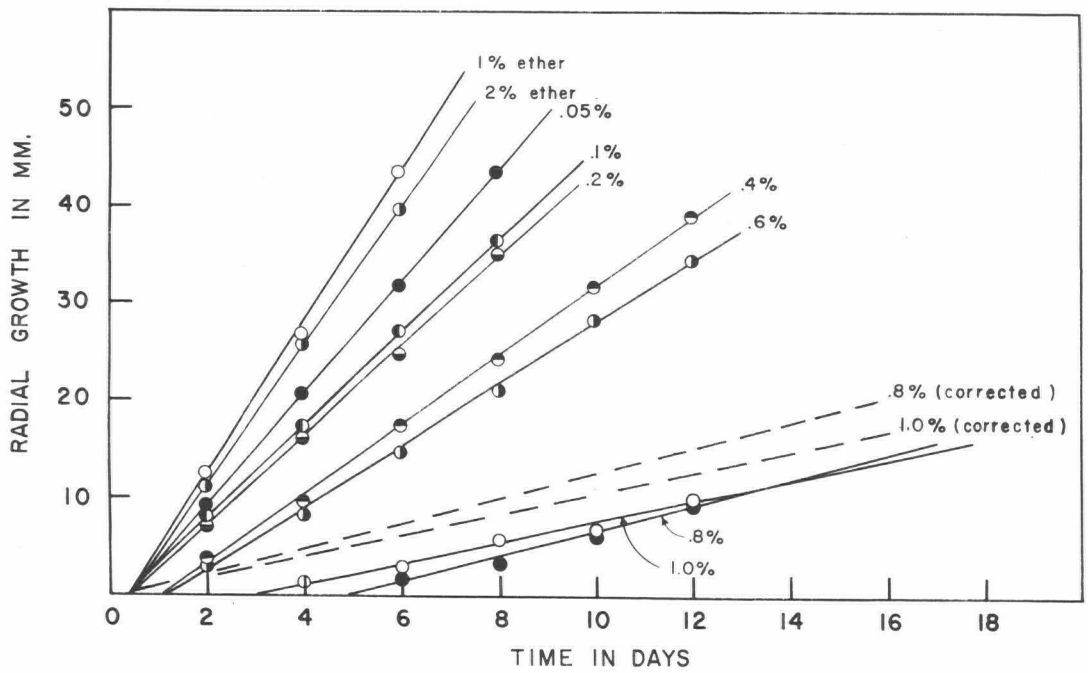
1% ether = control for 0.05%, .1% and .2% acids

2% ether = control for .4%, .6%, .8% and 1.0% acids

Broken line represents data corrected for rest period.

Figure 14.

Retardation of growth of Fomes annosus on malt agar containing different concentrations of free acids.


RADIAL GROWTH IN MM.
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
TIME IN DAYS
1% ether
2% ether
.05%
.1%
.2%
.4%
.6%
.8% (corrected)
1.0% (corrected)
.8%
1.0%

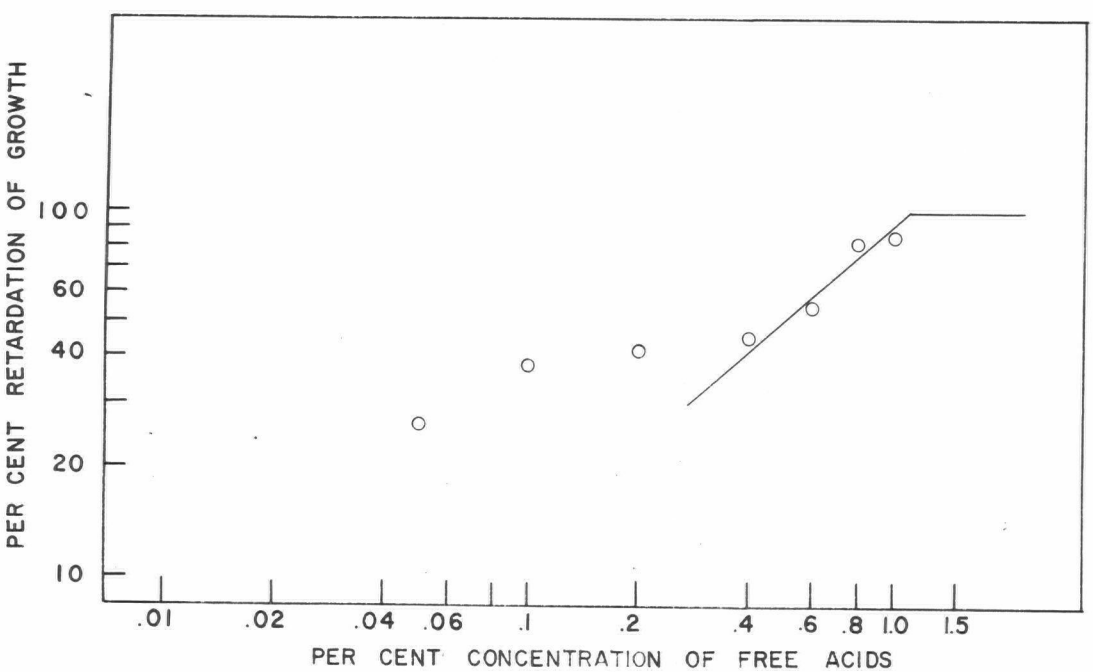

PER CENT RETARDATION OF GROWTH
100
60
40
20
10
.01
.02
.04
.06
.1
.2
.4
.6
.8
1.0
1.5
PER CENT CONCENTRATION OF FREE ACIDS

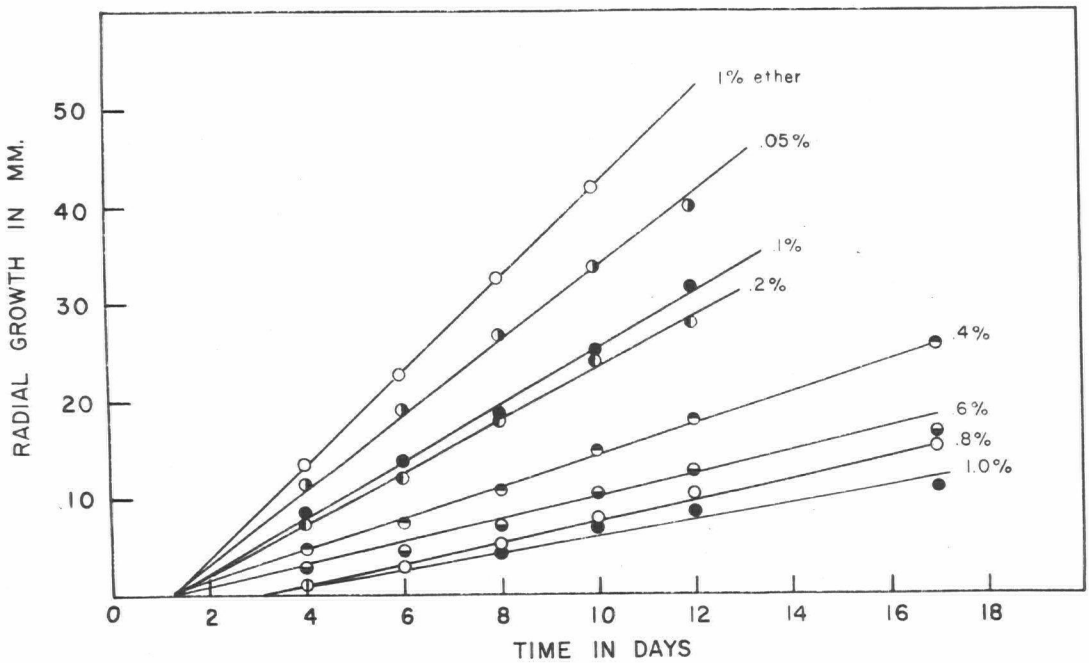
Figure 15.

Rate of growth of Lentinus lepideus on malt agar containing different concentrations of free acids.

1% ether = control

Figure 16.

Retardation of growth of Lentinus lepideus on malt agar containing different concentrations of free acids.


RADIAL GROWTH IN MM.
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
TIME IN DAYS
1% ether
.05%
.1%
.2%
.4%
.6%
.8%
1.0%

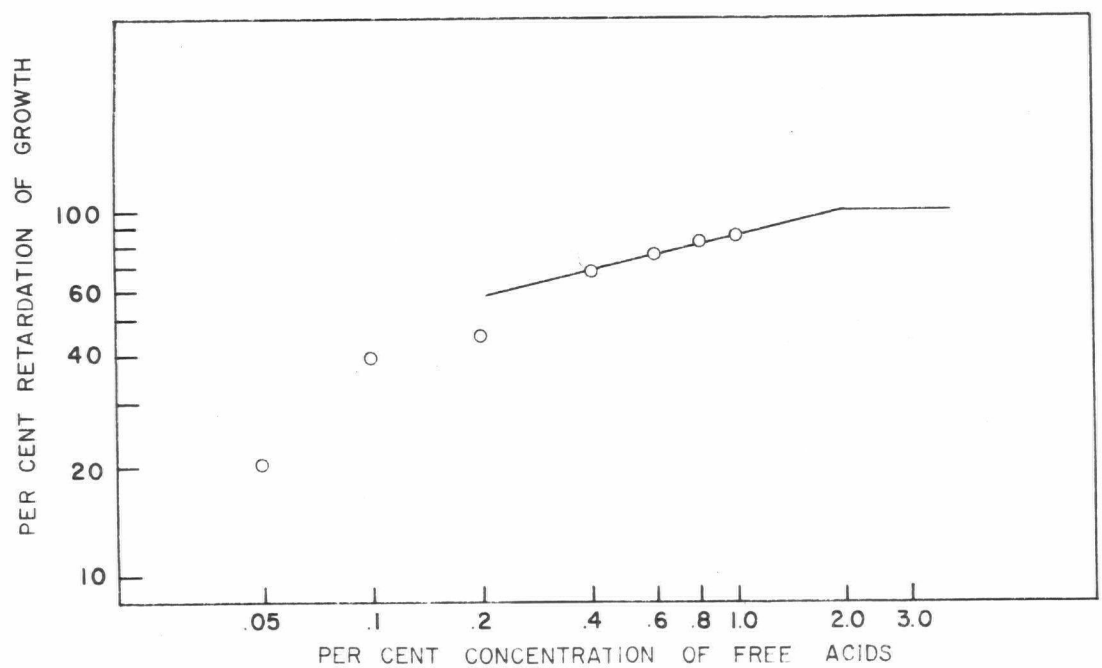
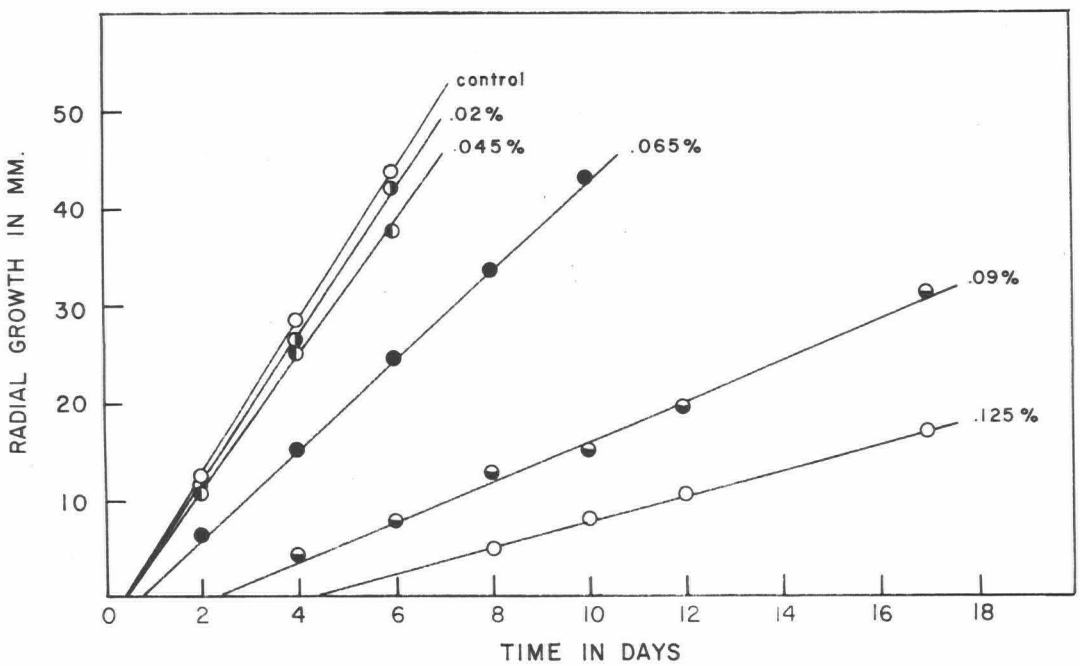

PER CENT RETARDATION OF GROWTH
100
60
40
20
10
.05
.1
.2
.4
.6
.8
1.0
2.0
3.0
PER CENT CONCENTRATION OF FREE ACIDS

Figure 17.

Rate of growth of Fomes annosus on malt agar containing different concentrations of zinc chloride.

Figure 18.

Retardation of growth of Fomes annosus on malt agar containing different concentrations of zinc chloride.


control
.02%
.045%
.065%
.09%
.125%
RADIAL GROWTH IN MM.
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
TIME IN DAYS

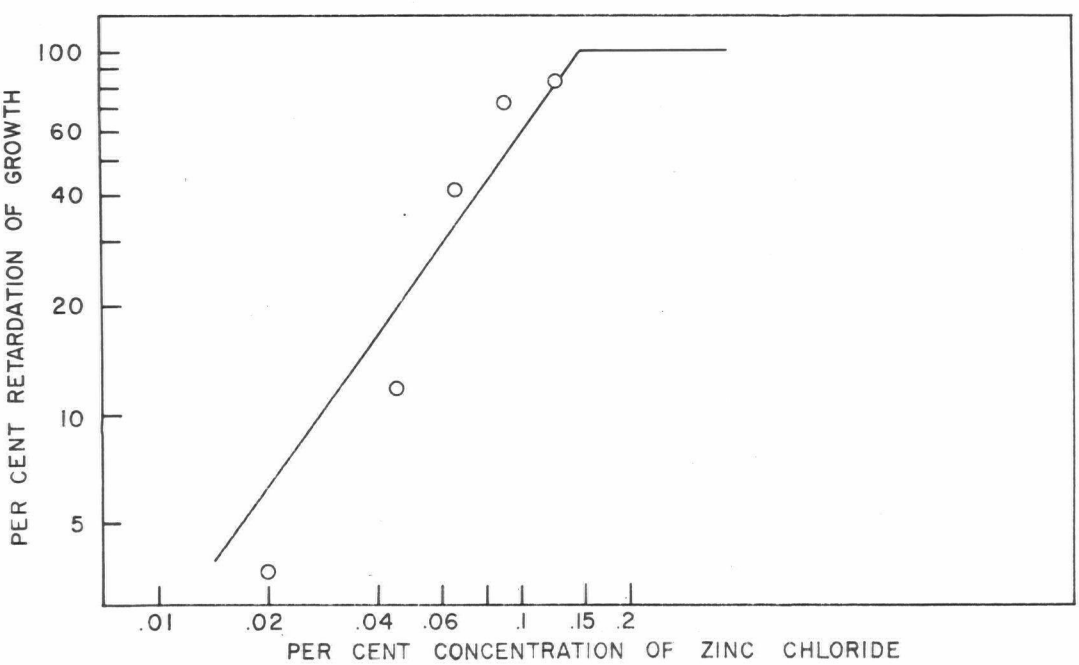
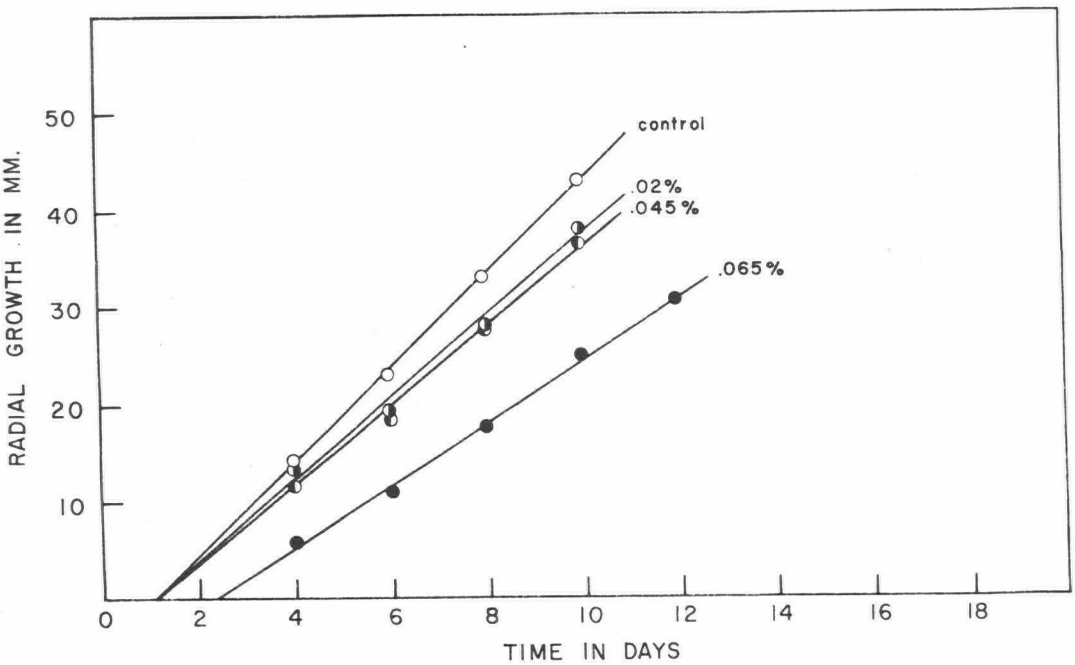

PER CENT RETARDATION OF GROWTH
100
60
40
20
10
5
.01
.02
.04
.06
.1
.15
.2
PER CENT CONCENTRATION OF ZINC CHLORIDE

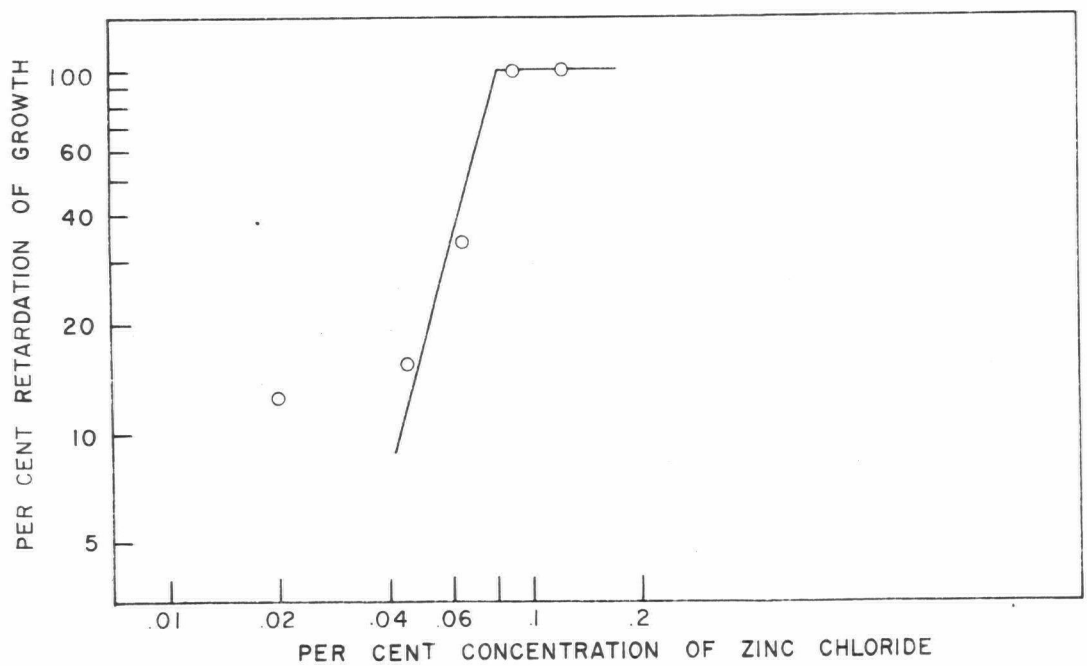
Figure 19.

Rate of growth of Lentinus lepideus on malt agar containing different concentrations of zinc chloride.

Figure 20.

Retardation of growth of Lentinus lepideus on malt agar containing different concentrations of zinc chloride.


RADIAL GROWTH IN MM.
50
40
30
20
10
0
2
4
6
8
10
12
14
16
18
TIME IN DAYS
control
.02%
.045%
.065%


PER CENT RETARDATION OF GROWTH
100
60
40
20
10
5
.01
.02
.04
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.1
.2
PER CENT CONCENTRATION OF ZINC CHLORIDE

APPENDIX B

Statistical analysis of weight losses in Douglas fir blocks exposed to *Poria incrassata* and *Lentinus lepideus*.

Per cent weight losses of extracted wood blocks exposed to action of Lentinus lepideus:

Ether	Acetone	Water	Control
54.58	49.01	36.01	39.24
51.68	55.29	44.33	43.93
43.78	57.40	50.80	43.63
53.12	52.36	50.90	23.43
55.79	47.05	41.28	37.70

Transforming these percentages to arc sin $\sqrt{\text{percentage}}$, they become:

	Ether	Acetone	Water	Control
	0.84	0.78	0.64	0.67
	0.81	0.84	0.73	0.73
	0.72	0.86	0.80	0.73
	0.82	0.81	0.80	0.50
	0.85	0.76	0.69	0.66
$\bar{x}$ =	0.81	0.81	0.73	0.66
$(x-\bar{x})^2$ =	0.0009	0.0009	0.0081	0.0001
	0.0000	0.0009	0.0000	0.0049
	0.0081	0.0025	0.0049	0.0049
	0.0001	0.0000	0.0049	0.0256
	0.0016	0.0025	0.0016	0.0000
	0.0107	0.0068	0.0195	0.0355

$$t = \frac{\bar{x}_1 - \bar{x}_2}{\sqrt{\left[\frac{(x - \bar{x}_1)^2 + (x - \bar{x}_2)^2}{n_1 + n_2 - 2}\right]\left[\frac{1}{n_1} + \frac{1}{n_2}\right]}}$$

Between ether and control:

$$t = \frac{0.81 - 0.66}{\sqrt{\left[\frac{.0107 + .0355}{8}\right]\left[\frac{2}{5}\right]}} = 3.12^*$$

$t_{.05} = 2.31$

$t_{.01} = 3.36$

Between acetone and control:

$$t = \frac{0.81 - 0.66}{\sqrt{\left[\frac{.0068 + .0355}{8}\right]\left[\frac{2}{5}\right]}} = 3.26^*$$

Between water and control:

$$t = \frac{0.73 - 0.66}{\sqrt{\left[\frac{.0195 + .0355}{8}\right]\left[\frac{2}{5}\right]}} = 1.33$$

Per cent weight losses of extracted wood blocks exposed to action of Poria incrassata:

Ether	Acetone	Water	Control
30.19	20.78	24.93	12.25
23.85	24.79	29.85	36.22
23.89	29.58	32.81	15.52
20.78	26.70	40.64	26.46
24.72	24.51	20.49	24.48

Transforming these percentages to arc sin $\sqrt{\text{percentage}}$, they become:

	Ether	Acetone	Water	Control
	0.58	0.48	0.52	0.35
	0.51	0.52	0.58	0.64
	0.51	0.58	0.61	0.41
	0.48	0.55	0.69	0.54
	0.52	0.52	0.46	0.51
$\bar{x}$ =	0.52	0.53	0.57	0.49
$(x-\bar{x})^2$ =	0.0036	0.0025	.0025	0.0196
	0.0001	0.0001	.0001	0.0225
	0.0001	0.0025	.0016	0.0064
	0.0016	0.0004	.0144	0.0025
	0.0000	0.0001	.0121	0.0004
	0.0054	0.0056	.0307	0.0514

Between ether and control:

$$t = \frac{0.52 - 0.49}{\sqrt{\left[\frac{.0054 + .0514}{8}\right]\left[\frac{2}{5}\right]}} = < 1$$

Between acetone and control:

$$t = \frac{0.53 - 0.49}{\sqrt{\left[\frac{.0056 + .0514}{8}\right]\left[\frac{2}{5}\right]}} = < 1$$

Between water and control:

$$t = \frac{0.57 - 0.49}{\sqrt{\left[\frac{.0307 + .0514}{8}\right]\left[\frac{2}{5}\right]}} = 1.25$$