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Title: BIOLOGICAL INFLUENCE OF RED ALDER ON PORIA WEIRII AND OTHER ROOT ROT PATHOGENS

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The chemical and microbial effects of red alder (Alnus rubra Bong.), a symbiotic fixer of nitrogen, on Poria weirii and other root-rot pathogens were investigated.

P. weirii was found unable to utilize nitrate as source of nitrogen for growth; it can, however, use ammonium and amino nitrogen. In the presence of nitrate ion, P. weirii and Douglas-fir leaves failed to induce nitrate reductase activity, whereas red alder leaves, Pythium debaryanum, Phytophthora cinnamomi, Neurospora crassa, Fusarium oxysporum, Fusarium avenaceum, Gliocladium roseum, Trichothecium roseum, and Verticillium albo-atrum showed positive nitrate reductase activity.

Soils under red alder and interplanted alder-conifer stands had higher percentages of bacteria, fungi and Streptomyces antagonistic against P. weirii than the soil of a pure conifer stand. P. weirii grew under acidic conditions ranging from pH 3.0 to 6.0;

maximum growth occurred at pH 6. 0. Growth did not occur at pH 6. 5, or above. The temperature range for growth was 15 to 25° C. Maximum development was at 20° C. No growth occurred at 30° C or above.

Poria weirii was found to possess extracellular oxidative enzymes, presumably phenoloxidases, which catalyzed the oxidation of catechol, DL-dopa, and hydroquinone, but not tyrosine, p-cresol, and p-phenylenediamine. Red alder leaves and roots possessed phenoloxidases, but these were lacking in Douglas-fir leaves and roots. Leaves of both alder and Douglas-fir showed peroxidase activity, but this was at much higher levels in alder than in fir.

Twenty-five phenolic compounds were tested individually in vitro at concentrations of 0. 5 and 2. 0 mM for effects on two isolates of P. weirii, T124 and T55. Growth of both isolates was strongly inhibited in a medium containing coumarin, 4-hydroxycoumarin, or 7-hydroxycoumarin at either concentration, but o-catechol, salicylic acid, benzoic acid, ferulic acid, o-coumaric acid, and phenylacetic acid were inhibitory only at the higher concentration. The remaining compounds either inhibited only one isolate, had no effect, or stimulated growth of the fungi. Chlorogenic and gallic acids at 2. 0 mM concentration which stimulated the growth of P. weirii T-124 also stimulated the endogenous respiration of this isolate, while phenylacetic acid, salicylic acid, and coumarin which at 2. 0 mM

concentration were inhibitory to growth of T124 depressed the endogenous respiration of this isolate. Linoleic acid which occurs in red alder was inhibitory to both P. weirii and Fomes annosus.

Analysis of ethanolic extracts showed a higher phenolic content (tannins) in roots, fresh leaves, litter and soil of red alder than those of Douglas-fir. Tannins in alder-conifer soil were higher than in conifer or alder stand soil. Growth experiment showed that tannic acid in higher concentrations inhibited growth of P. weirii on malt agar.

Chemical analysis of ethanolic extracts showed that red alder roots contained ferulic acid and vanillic acid, while roots of Douglas-fir contained vanillic acid only. Litter of both kinds of trees contained vanillic acid. Fresh red alder leaves contained caffeic acid and vanillic acid. Soils from red alder and alder-conifer stands also contained vanillic acid, but only trace amounts were found in conifer soil.

A compound inhibitory to P. weirii was extracted with petroleum ether from red alder fresh leaves. It had absorbance peaks in methanol at 475 mμ, 442 mμ, and 260 mμ. It appeared dull green under ultraviolet light and bright yellow in daylight; it had a Rf value of 0.58 on silica gel F_{254} with a benzene-ethyl acetate (9:1) solvent system. This compound was not found in leaves of Douglas-fir.

Biological Influence of Red Alder on *Poria weirii* and Other Root Rot Pathogens

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BIOLOGICAL INFLUENCE OF RED ALDER ON PORIA WEIRII AND OTHER ROOT ROT PATHOGENS

INTRODUCTION

Red alder (Alnus rubra Bong.), often called Oregon alder, grows in southeastern Alaska (Ya Kutata Bay), British Columbia, Washington, northern Idaho, Oregon and the Santa Ynez mountains of California. The tree grows best at low elevation where the climate is mild and the rainfall heavy. The U. S. Forest Service estimates that about 892, 000 acres of commercial forest land in Oregon and Washington are occupied by stands predominantly red alder; the area on which the tree is intermingled in stands predominantly conifers is far greater (Worthington et al., 1962). The production of red alder lumber in the United States amounted to 18. 9 million board feet in 1954. About two-thirds of this volume or 12. 4 million board feet was produced in Oregon (Betts, 1960). Aside from its commercial value, red alder is of special importance because of its ability to increase soil nitrogen through symbiotic fixation with microorganisms contained in root nodules. Its possible importance to forest culture is comparable to that of leguminous plants in agricultural crop production.

General observations in Washington and Oregon over the last several years have indicated that Poria weirii is less of a disease

problem in conifer stands containing red alder than in pure conifers. Economic losses from P. weirii alone in the Pacific Northwest were estimated about 32 million cubic feet annually (Childs and Shea, 1967). Unless research efforts are made proportionate to the huge value at stake, the diseases will continue to cause heavy losses indefinitely. Direct control of root diseases in forestry is relatively ineffective, and in any event far too costly for general use. Experience in agriculture suggests that satisfactory control of root rot problems of forest trees is most likely to be obtained by indirect methods, that is, by modifying environmental conditions to make them unfavorable for the pathogens. The possibility that alder acts as a biological control agent against this serious root pathogen of western North American conifers merits high priority attention, both because no feasible mechanical or chemical methods are in prospect for reducing severity of established infections and because of the urgency of preventing further environmental pollution from widespread application of chemicals. This study was undertaken to indicate if red alder may have possible effects in reducing the incidence of root-rot diseases of conifers caused by P. weirii and other soil-borne pathogens.

LITERATURE REVIEW

Microbial and Chemical Properties of Soils Under Red Alder, Conifers and Alder-conifer Mixed Stands

Chen (1965) showed that plate counts of molds and bacteria were affected by forest type. Molds were least abundant in the soil under red alder, and were always most numerous in soil under conifers. Conifer soil gave highest counts of bacteria; alder-conifer soils showed lowest numbers. The percentage of Streptomyces was greatest under the mixed stand and generally was lowest under conifers. The preponderance of Streptomyces in the mixed stand was suggested by Bollen et al. (1964) to be one of the important factors in reducing pathogens attacking conifer roots. Chen also pointed out that soil under red alder had the lowest pH value ranging from 3.9 to 5.2; under conifer the pH ranged from 4.7 to 5.9; and under the mixed stand, from 3.9 to 5.7. Nitrate nitrogen was very high in alder and the mixed stand soils with a maximum of 270 ppm, while for conifer it was only 75 ppm. He suggested that high nitrate nitrogen as well as acidity under the mixed stand was important in inhibiting soil-borne root rot pathogens.

Beneficial Effect of Alder in Mixed Stands

Although alder has been considered a weed tree and in many respects undesirable, various workers have shown that it is of great value as a nurse crop to more valuable tree species. Wiedemann (1942) reported that pine roots in association with grey alder were more effective than pure pine stands in penetrating deeper soil. Duchanfour (1948) recommended admixture of pine with Alnus incana and A. cordata on humid mountain soil and on poor acid soil. Masson (1954) suggested alder with oak in Belgium to improve the soil. Mixed stands of Populus species with A. glutinosa have been recommended to increase yield (Gambi, 1958; Red'ko, 1958; Pogrebnjak, 1960). Johnson (1917) pointed out that red alder stimulated growth of Douglas-fir in the Siuslaw National Forest in Oregon. It revivified the depleted soil, making possible the restoration of desirable tree species which once covered this region; however, he did not give experimental data to support this.

Tarrant and Miller (1963) found that soil under a mixed 30-year stand of Douglas-fir and red alder had a 50 percent greater nitrogen content than soil under pure alder or Douglas-fir stands. Organic matter also was significantly greater in the soil of the mixed stand. The top 36 inches of soil contained 1,665 lbs more nitrogen per acre than soil under red alder; this represented an

average annual accumulation of 64 lbs per acre attributed to the alder, 36 lbs more per acre than under Douglas-fir.

Bornebusch (1946) reported that when leaves of various species were placed on a good forest mull, those of alder decomposed most rapidly while those of Norway spruce and Douglas-fir decomposed more slowly. He also found that decomposition of cellulose was stimulated by the presence of various species of alder leaves in the soil. Norway spruce needles inhibited cellulose decomposition on mull, but were stimulating on mor, whereas needles of Douglas-fir inhibited cellulose decomposition on mor and did not have a favorable effect upon rate of cellulose decomposition.

Mikola (1958, 1959) showed that A. glutinosa litter increased weight of pine seedlings, while litter from seven other tree species did not. The beneficial effect of alder litter was due to the higher nitrogen content; the harmful effects of other litter were attributed to low nitrogen content. Similar results were reported by Schalin (1966), who treated pine and spruce plots with litter of A. incana, and by Wollum and Youngberg (1964) who found that A. rubra litter improved growth of pine seedlings whereas Douglas-fir litter was depressive.

Utilization of Nitrate Nitrogen by Fungi

Nitrate is an excellent source of nitrogen for many fungi.

However, among the basidiomycetes nitrate users are not common. Melin (as cited by Fries, 1955) found that only a few mycorrhizal fungi utilized nitrate. Leonian and Lilly (1938) found that Collybia tuberosa and Lentinus tigrinus grew well on a nitrate-containing medium. LaFuze (1937) found that Fomes pinicola could use nitrate for growth. Lindeberg (1944) and Lindeberg and Molin (1949) noticed that only two among 15 different Marasmius species had this ability. Lilly and Barnett (1951) published a list of nitrate-utilizing fungi, but it contained only a few basidiomycetes. Hacskaylo et al. (1954) tested 14 wood-destroying basidiomycetes for nitrate utilization; only a few could grow with this nitrogen source, and only to a limited extent; Polyporus distorlus developed better, though slowly. Fries (1955) found that Coprinus fimetarus II and C. ephemerus grew well with nitrate but it was less favorable for C. micaceus and C. narcoticus. Norkrans (1950) observed that Tricholoma nudens could utilize nitrate nitrogen.

Polyphenoloxidases and Peroxidase in Plants and Their Relation to Disease Resistance

The importance of polyphenoloxidases in the phenolic defense mechanism was emphasized by Rubin and Aksenova (as cited by Rubin and Artsikhovskaya, 1963). They showed that in Phytophthora infected potato, polyphenoloxidases played an important role in the

hypersensitive response of resistant varieties. Under the action of Phytophthora, polyphenoloxidases of immune varieties underwent considerable activation directly after penetration of the hyphae into the living cell. The dark-colored products of phenol oxidation accumulated in the infected cell resulting in the inhibition of respiratory dehydrogenases, causing death of plant cells containing these products. Polyphenoloxidases showed little change in activity upon the penetration of hyphae in susceptible varieties, while hydrogenases became considerably activated. This allowed the infected cells to continue normal respiration and further development of the parasite at the same time. Fuchs (1956) and Valle (1957) further found that Phytophthora resistance was reversed by copper enzyme inhibitors that interfered with formation of toxic quinones via polyphenoloxidase.

Phloridzin, a polyphenolic glucoside found in the bark and leaves of apple, is hydrolyzed to phloretin in the presence of β-glucosidase, and its further oxidation to intermediates appears to be responsible for resistance to scab caused by Venturia inaequalis. Some workers attributed varietal resistance to quantitative differences in phenolic content of the leaves (Kirkham, 1959). A polyphenoloxidase inhibitor--4-chlororesorcinol--has been observed to induce sporulating lesions on resistant varieties (Noveroske, 1962), indicating that phloretin oxidation was involved in host resistance. However, Barnes and Williams (1961) claimed inhibition by

Preformed Phenolic and Other Compounds as the Resistance Factors in the Healthy Plant

It is now generally agreed that phenolic compounds are involved in some manner in the mechanism of resistance of some plants against infection by viral, bacterial and fungal pathogens (Farkas and Király , 1962; Cruickshank and Perrin, 1964; Hare, 1966) and against harmful insects (Rubin, 1967). The best known example for the protective role of preformed phenolics is in the onion-Collectorichum circinans complex. Link et al. (1929) pointed out that colored varieties of onion resistant to C. circinans (Berk.) Vogel. contained more protocatechuic acid in their scales than did varieties with white scales, which are susceptible. Link and Walker (1933) also found that catechol was present in scales of a resistant onion variety. They showed that these two compounds suppressed the growth of the fungus in vitro (Walker and Link, 1935). A case similar to that described by the Walker school was claimed to hold true for the resistance of onion bulbs to Diplodia natalensis by Ramsey, Heiberg and Wiant (1946). However, this study lacks detailed chemical analysis.

In some other cases phenolics localized in nonliving protective layers of plant organs play a fungistatic role in preventing infection at the portal of invasion. Clauss (1961) and Schneider (1952) found that the seed coat of pea seeds of varieties resistant to causal

organisms of root diseases contained higher amounts of phenolic compounds. One of the main components having clear-cut antifungal properties was identified as belonging to the leucoanthocyanins. An example for the role of the pre-infectional level of phenolic content in resistance is the common scab of potato (Johnson and Schaal, 1952). They tested a high number of potato varieties and strains of known scab resistance for the phenol content of their periderm by use of the $FeCl_3$ test. A good correlation was found between resistance and green color formation. Detailed paper chromatographic and spectrophotometric studies gave evidence that the compound involved was chlorogenic acid. They emphasized the importance of the unequal distribution of chlorogenic acid, the phenolic being localized mainly around the lenticels where infection occurs. Chlorogenic and caffeic acids were reported by Kuć et al. (1956) to be closely associated with the natural immunity of white potatoes from attack by Helminthosporium carbonium.

There have been relatively few reports on phenolic defense mechanisms in woody plants. Pyrocatechol was identified as one of the main substances isolated from aspen bark which inhibited Hypoxylon pruinatum canker (Hubbes, 1962). Species of chestnut resistant to blight (Endothia parasitica) showed more water soluble tannins in the bark (Nienstead, 1952), and tannins from resistant Chinese chestnut were much more toxic to the blight fungus than

tannins from susceptible species. Various tannins tested by Lewis and Papvizas (1967) showed inhibition of growth of Fusarium solani f. phaseoli and Verticillium albo-atrum. That tannins are involved in disease resistance mechanisms has also been shown by Somers and Harrison (1967).

Kurth and Becker (1953) isolated linoleic acid, an unsaturated 18-carbon-atom fatty acid, from bark of red alder, and Honkanen and Virtanen (1960) reported that this acid inhibited Fusarium nivale. However, a search of the literature revealed that little previous work has been done on the effects of fatty acids on growth of fungi, particularly on the root rot fungi of woody plants. The systematic investigation of Kiesel (1913) at the Pasteur institute revealed that the fatty acids containing from 1 through 11 carbon atoms inhibited germination of spores, formation of mycelium, and production of conidia by Aspergillus niger. The work of Kitajima and Kawamura (1931) with Poria vaporaria and Paxillus panuoides essentially confirmed Kiesel's findings, but they found that the toxicity increased up to 12 carbons, and that the unsaturated fatty acids were more toxic than the corresponding saturated acids. Dicarboxylic acids were found to be almost non-toxic. The studies of Baechler (1939) on wood-destroying fungi, of Hoffman et al. (1939) on molds, and of Rigler and Greathouse (1940) on Phymatotrichum omnivorum substantiated those of earlier workers. However, ability to grow on

fatty acids as sole carbon source is not unique among the fungi. Mukherjee (1952) reported the utilization of butyric acid by A. niger. Stearic acid and oleic acid are utilized by Mucor mucedo (Stern et al. 1954). Both long and short chain fatty acids are used for growth and energy by Spicaria violacea (Johnson, 1957, 1958; Johnson and and Dixon, 1959) and by Cunninghamella echinulata (Lewis and Johnson, 1966, 1967, 1968).

EXPERIMENTAL METHODS AND MATERIALS

Nitrogen Utilization by Poria weirii

Poria weirii T124 was grown on liquid media (Jennison, Newcomb and Henderson, 1955) with potassium nitrate, ammonium chloride, and L-asparagine as respective sources of nitrogen. Each form of nitrogen was sterilized by filtration through an ultrafine porosity fritted disc, and was included at each of three concentrations: 10, 100, and 1,000 ppm nitrogen in 50 ml medium. In each case four replicate 125-ml flasks were inoculated with 4 mm agar disks cut from margins of colonies of P. weirii growing on malt agar. Cultures were grown for 40 days at room temperature (24° - 28°C), after which the mycelial mats were collected, dried overnight at 105°C and weighed. The procedure of extraction of nitrate reductase was essentially that devised by Nason and Evans (1953), or by Egami and Taniguchi (1963); the enzyme activity of the cell-free extract was determined by the colorimetric test for nitrite.

Detection of Soil Microorganisms Antagonistic to P. weirii

Soils were collected from Cascade Head Experimental Forest near Otis, Oregon. Before sampling, the surface layer of decomposed plant materials was removed. Samples were taken from five

different locations in each plot, each sample representing a mixture of five subsamples taken about two feet away from the tree, at six inches depth. In the laboratory, the samples were immediately screened through a ten-mesh sieve. Oven-dry weights of screened samples were determined after drying 24 hours at 105° C.

Serial dilutions of soil were prepared in sterile distilled water, starting with an initial dilution of 20 g soil (oven dry basis) and sufficient water to make 100 ml, giving a 1:5 dilution. For fungi, 1.0 ml aliquots of a 1:5000 dilution were plated out in four replicates with Martin's (1950) peptone-dextrose rose bengal agar containing 30 ppm streptomycin sulfate. For Streptomyces and bacteria, 1.0 ml aliquots of a 1:50,000 or 1:500,000 dilutions were plated in a similar manner with sodium albuminate agar. Developing colonies were transferred to a suitable medium for growth and each was tested for antagonism against P. weirii T-124. In another experiment, cultures of P. weirii grown in shake flasks were harvested and washed two times with sterile distilled water. The mycelium was then homogenized in a Waring blender. Two ml of mycelial suspension were then plated with two percent water agar. Five ml of melted malt agar were then layered on the surface of the water agar culture of P. weirii. A sterile sharp-tip tooth-pick was used to inoculate the malt agar surface with fungi developed on the soil dilution plates. For Streptomyces inoculations, another 5 ml Czapek solution agar were poured on the

solified malt agar layer. The tooth-picks were discarded after each inoculation. The inoculated areas were so arranged that the distance between inoculations was about 15 mm. The malt agar supported the growth of P. weirii into a thick mycelial mat which made the inhibition zone around the colonies more distinct (Plate I). The number of antagonistic microorganisms per gram soil was calculated by multiplying the average number per dilution plate with the appropriate dilution factor.

pH and Temperature Studies on Growth of Poria weirii

The liquid medium developed by Trione (1964) was used, except that 10 g of glucose per liter was substituted for 20 g of sucrose. The medium was divided into twelve 200-ml portions and the pH of each was adjusted with HCl or NaOH to provide a series ranging from pH 3.0 to 7.5 at 0.5 intervals. After sterilization by filtration through an ultrafine porosity fritted disk, 50 ml of medium at each pH were added aseptically to four replicate, sterile, 150-ml Erlenmeyer flasks. A 4 mm plug of inoculum, cut from a seven-day-old malt agar culture of P. weirii, then was added. After incubation at 25°C for 30 days, mycelia were harvested, freed of the agar inoculum plug, dried overnight at 105°C and weighed.

For study of temperature effects, similar procedures were followed, except that the medium was adjusted to pH 6.0 and

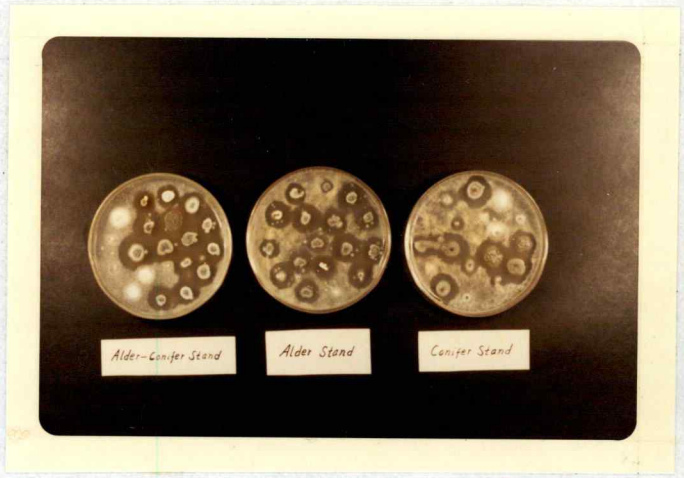


Plate 1a. Soil fungi antagonistic against P. weirii.

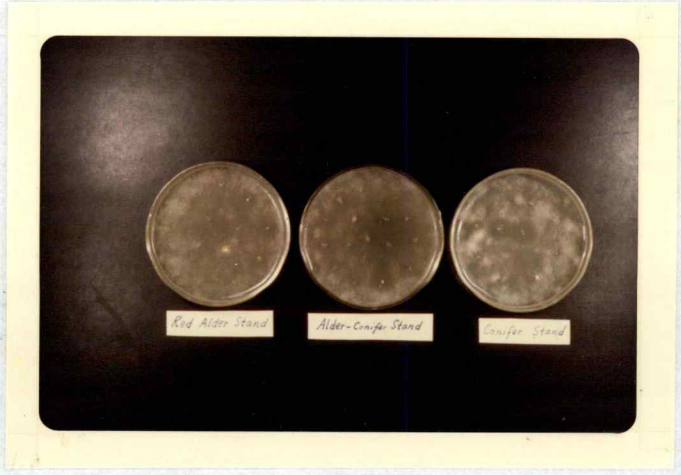


Plate 1b. Streptomyces antagonistic against P. weirii.

sterilized by autoclaving; five replicates were used at each temperature in the range of 5 to 35° C at 5° intervals. As a quick check on temperature effects, five malt agar plates inoculated with similar mycelial plugs were placed in each incubator along with the flasks of liquid medium. Colony diameters of these were measured at three day intervals up to 12 days after inoculation.

Detection of Extracellular Phenoloxidases Produced by P. weirii

A culture of P. weirii was grown in 250 ml of liquid synthetic medium (Trione, 1964) in which 10 g glucose was substituted for sucrose. After three weeks of growth, 10 ml aliquots of the sterile medium were pipetted into each of 26 Erlenmeyer flasks. The medium in 12 of the flasks was boiled to inactivate enzymes for controls. Meanwhile, solutions of p-cresol, DL-dopa, catechol, p-phenylenediamine, tyrosine, and hydroquinone were prepared at concentrations of 2.75 mg/ml double-distilled water and sterilized by glass filtration through ultrafine porosity fritted discs. Two ml of each phenolic compound were then added to each of two flasks containing boiled and unboiled aliquots of medium on which P. weirii had been grown, while similar amounts of distilled water alone were added to two flasks of unboiled medium as additional controls. Brown to black discoloration of the mixtures indicated activity of enzymes on the specific phenols.

Extraction of Phenoloxidases from Red Alder and Douglas-fir

The extraction procedure was essentially the same as that of Badran and Jones (1965). Four grams of leaves were collected from two-year-old, greenhouse-grown trees and washed in cold, redistilled water. The leaves were cut into small fragments while immersed in 100 ml 0. 1 M potassium phosphate buffer at pH 6. 5, containing one percent polyethylene glycol (PEG), molecular weight 4, 000 (Carbowax 4000, Union Carbide Chemicals Company). The fragments were vacuum infiltrated for 30 minutes, then removed and added to another 40 ml PEG-buffer solution. This mixture was homogenized in an Omni-Mixer at maximum speed for three minutes, after which the homogenate was poured into 80 ml cold acetone (-20°C), and centrifuged at 1, 200 x g for ten minutes. The precipitate was immersed for 30 minutes in 20 ml of 0. 1 M potassium phosphate buffer, pH 6. 5, to solubilize the enzymes, and centrifuged at 3, 000x g for ten minutes. The supernatant was decanted and recentrifuged at 18, 000x g for ten minutes. The final supernatant was then tested for polyphenoloxidase activity. A similar procedure was used for roots.

The assay mixture contained 0. 1 ml enzyme extract and 2. 9 ml or 10^{-3} M substrate-catechol, chlorogenic acid or DL-dopa. The reaction was followed by measuring light absorption at 15-second

intervals in a Beckman DB spectrophotometer at 400 mμ for chlorgenic acid and catechol and at 495 mμ for DL-dopa. This 0. 1 ml enzyme extract represented the enzyme activity per 18 mg fresh leaf tissue, or 20 mg fresh root tissue.

Extraction of Peroxidase from Red Alder and Douglas-fir

Six grams of leaves of each species were collected from two-year-old greenhouse-grown trees. The leaves were washed in cold, redistilled water and stored in a cold room at -17° C for 18 hours. They were then homogenized with 50 ml buffer at pH 7. 0 (McIlvaine, 1921) containing 10^{-2} M cysteine and 6 g of dry polyclar AT (Loomis and Battaile, 1966) in an Omni-Mixer at maximum speed for three minutes. The homogenate was filtered through fine-mesh nylon cloth and the filtrate centrifuged at 3, 000 x g for five minutes. The clear supernatant was then tested for peroxidase activity. The assay mixture consisted of 0. 5 ml enzyme extract, 0. 1 ml of 0. 2 M pyrogallol, 1. 0 ml McIlvaine's buffer, pH 7. 0, 0. 5 ml of 0. 01 M H_2O_2 and 0. 9 ml redistilled water. Blanks for the spectrophotometric analysis were composed of the same assay mixture, but one lacked enzyme extract and the other, H_2O_2. Absorption at 430 mμ was measured at intervals of 15 seconds in a Beckman DB spectrophotometer. This 0. 5 ml enzyme extract represented the enzyme activity per 60 mg fresh leaf tissue.

Reduction of Nitrate to Nitrite by Red Alder and Douglas-fir

Ten grams of leaves were collected from Cascade Head Experimental Forest (maintained by the U. S. Forest Service, Pacific Northwest Forest and Range Experiment Station), Oregon. The leaves were surface-sterilized by dipping into 2. 65 percent sodium hypochlorite solution for five minutes and washed two times with sterile distilled water. They were then immersed for four days at room temperature in 600 ml Hoagland solution I (Hoagland and Arnon, 1938), which had been diluted to one-fourth strength with sterile distilled water. This step was taken on the basis that reduction of nitrate requires the enzyme nitrate reductase (Townsend and Blatt, 1966), whose production is induced in the presence of nitrate ions (Beevers and Hageman, 1963; Raghavan and Torrey, 1964; Rijien, 1958 and 1960). Two ml of streptomycin sulfate solution (10 mg/ml) were also added to the solution to minimize bacterial growth. Nitrite released into solution was tested by adding 1. 0 ml of one percent sulfanilamide and 1. 0 ml 0. 02 percent N-(1-naphthyl)ethylenediamine hydrochloride to 2 ml test solution. After 20 minutes, the color density was read at 540 mμ on the Beckman DB spectrophotometer. Hoagland solution without leaf inoculation, and sterile distilled water with leaf inoculation were used as blanks.

Studies on Growth of *Poria weirii* in *vitro* Containing Various Phenolic Compounds

Twenty-five compounds were tested for ability to inhibit growth of two isolates of *P. weirii*, T 55 and T 124. Trione's (1964) synthetic medium was used, modified by incorporating 10 g glucose instead of 20 g sucrose and by not adjusting the post-autoclaving pH 5. 5. The respective compounds were dissolved in 50 percent ethanol which was then adjusted to pH 5. 5 with NaOH. Sixteen 250-ml Erlenmeyer flasks, each containing 50 ml of basal medium, were used in testing each compound. A 0. 5 mM concentration of the compound was attained in eight flasks by adding 0. 25 ml of its ethanolic solution; in the other eight, 1. 0 ml of solution was added for a concentration of 2. 0 mM. For controls, 0. 25 or 1. 0 ml of 50 percent ethanol were added respectively to each of eight flasks of medium. Plugs of inoculum 4 mm in diameter, taken from the edges of 14-day-old colonies of a given isolate of *P. weirii* grown on malt agar plates, were transferred into four flasks of each compound at each concentration, as well as into controls; flasks were then incubated at 20° C for 22 days for isolate T 124 and 29 days for the slower growing T 55. After harvest, mycelia were weighed after drying 48 hours at 85° C. Final pH of the medium was determined for each flask.

In metabolic studies, respiration was measured by direct methods (Umbreit, Burris and Stauffer, 1964). Mycelium of 16-day-old shake culture of P. weirii T-124 grown in modified Trione's synthetic medium was centrifuged and washed three times with sterile distilled water and then homogenized in sterile distilled water. Two ml of mycelium suspension (equivalent to 1. 3 mg dry weight of mycelium) were pipetted into the main compartment of the reaction vessel which contained 0. 5 ml of 0. 067 M phosphate buffer, pH 5. 5. Two-tenths ml of 20 percent KOH was added to its central well. Five-tenths ml of the 2. 0 mM phenolic compounds to be tested was added to the side arm of the vessel. Duplicate flasks of each treatment were attached to calibrated manometers and placed in a constant temperature water bath at 20° C. The flasks were allowed to equilibrate for 15 minutes before the phenolic compound was tipped into the main compartment of the vessel.

Studies of Linoleic Acid on Growth of P. weirii and Fomes annosus

Linoleic acid (95-97 percent, K and K Laboratories, Inc., Plainview, New York) was converted to its potassium salt with KOH in absolute ethanol. The salt was precipitated; washed with anhydrous, peroxide-free ether; dried in a vacuum dessicator; and then dissolved at concentration of 0. 1 percent in Trione's (1964) medium modified by substitution of 10 g glucose for 20 g sucrose,

Na_2SO_4 for $MgSO_4 \cdot 7H_2O$, and KCl for $CaCl_2$. The resulting medium was adjusted to pH 5.5 with 0.1 N HCl, sterilized by filtration through ultrafine porosity fritted discs, and added to similar but linoleic acid-lacking medium in quantities necessary to total 7.0 ml in 150 x 6 mm test tubes. The final salt concentration per ml in tubes became 0.1, 0.3, 0.5, 0.7, 1.0, 1.5, 2.0, and 4.0 mg.

Shake cultures of P. weirii T-124 and F. annosus were meanwhile grown on Trione's medium for 20 and 24 days, respectively. The mycelia were then harvested, washed three times in sterile distilled water, and homogenized in sterile distilled water. One-tenth ml of mycelial suspension (equivalent to 0.175 mg sucrose) of each fungus was added to each of four tubes of each concentration of linoleic acid salt. Cultures were incubated at 20°C for 17 days for F. annosus and 22 days for P. weirii. Final weight of mycelium in each tube was determined by the microchemical method of Lu et al. (1959). Data were subjected to a polynomial regression analysis.

Extraction and Identification of Phenolic Compounds from Plants and Soils

Twenty-five grams of lyophilized roots, litter, and leaves of Douglas-fir and red alder were finely ground and then exhaustively extracted with 300 ml 95 percent ethyl alcohol in a Soxhlet apparatus. The solvent was removed under reduced pressure in a flash

evaporator. The residue was lyophilized to dryness, after which it was taken up with boiling water; total phenolics were then determined colorimetrically according to Folin and Denis (1915), as modified by Goldstein and Swain (1963), using tannic acid as the standard. To 1.0 ml sample solution 1.0 ml Folin-Denis reagent was added. After three minutes 1.0 ml 1 N Na_2CO_3 was added and the optical density was read one hour later at 725 mμ.

To identify the individual phenolic compounds in the extracts, the procedures of Bohm and Towers (1962) were employed. The alcoholic extractive was acid hydrolized in 2 N HCl on a steam bath for one hour; or alkali hydrolized in 2 N NaOH at room temperature for at least 12 hours, after which it was acidified with HCl. The acid or alkali hydrolysate was continuously extracted with ether for at least six hours. The ether extracts were then evaporated to dryness, taken up in a few ml of hot 95 percent ethanol, and applied as spots to steamed cellulose-silica gel G plates (Sumere et al. 1965). The development of the chromatograms was carried out at room temperature in a solution of chloroform, acetic acid, and water (4:1:1). Phenolic compounds were detected on plates under ultraviolet light (350 mμ) both before and after spraying the thin layers with 2 N sodium hydroxide. After this, the position of the phenolics was revealed by spraying with diazotized p-nitroaniline (Procházka, 1966). The colors of the compounds were matched with a Reinhold

color atlas (Kornerup and Wanscher, 1962).

Procedures for extraction of phenolic compounds from soil were essentially those devised by Whitehead (1964). Two hundred and fifty grams moist fresh soils of conifer, red alder, and alder-conifer mixed stands from the Cascade Head Experimental Forest were shaken on a Miller paint mixer for three hours with 2.5 g calcium oxide and 250 ml distilled water. The resulting filtrate was acidified to pH 2 with sulfuric acid, treated with freshly precipitated zinc ferrocyanide to remove fatty material (Moir, 1935; Hamence, 1944), and filtered again. Acidity was then readjusted to pH 2 and the filtrate extracted with an equal volume of peroxide-free ether as described by Hamence (1944). The ethereal solution was washed with water, dried with anhydrous sodium sulfate and evaporated to a small volume. Confirmatory evidence of identification was provided by the colors given on staining developed cellulose-silica gel G plates in chloroform-acetic acid-water (4:1:1) with diazotized p-nitroaniline as described above.

Petroleum Ether Extraction of Red Alder and Douglas-fir Leaves

Twenty-five grams of lyophilized fresh leaves which were collected in August, 1968, from Mary's Peak, west of Corvallis, Oregon, and had been ground into small particles by means of a Micro Mill were exhaustively extracted in Soxhlet apparatus with

250 ml petroleum ether. After extraction, the solvent was evaporated to dryness at room temperature. The residues obtained were dissolved in a few ml absolute alcohol and spotted on a silica gel F_{254} plate, 20 x 5 cm, 10 mm from the base (E. Merckag, Darmstadt, Germany) by means of capillary tubes. The solvent, benzene-ethyl acetate (9:1), was added to the tank at least half an hour before a plate was developed to allow the atmosphere to become saturated. After development, the plate was dried at room temperature. The separation of the compounds was examined in the daylight as well as under ultraviolet light (350 mμ).

Fractionation of leaf extracts by column chromatography was achieved by use of silica gel G packed to 20 cm length in a 3 x 45 cm chromatographic tube. Five ml extracts, which represented 4.15 g leaf tissue, were applied to the column. Elution with benzene-ethyl acetate (9:1) solvent, at about 30 ml/hour, gave a yellow band followed by dark green and light green bands. The yellow band eluants were collected and evaporated to dryness at room temperature. The 12.3 mg of yellow compound obtained was dissolved in 1.5 ml absolute alcohol and added to modified Trione's medium giving final concentrations of 0.04, 0.07, 0.10, and 0.14 mg/ml. A separate series using absolute alcohol in modified Trione's medium was used as a check. A shake culture of P. weirii T-124 grown on Trione's medium for ten days was harvested, washed three times and

homogenized in sterile distilled water. One-tenth ml of this mycelial suspension of the fungus was added to each of four tubes of each concentration of the yellow compound. Cultures were incubated at 20°C for 11 days. Final organic matter of mycelium in each tube was determined by the microchemical method of Lu et al. (1959).

RESULTS AND DISCUSSION

Nitrogen Assimilation by *Poria weirii*, Other Fungi, and Plants

Poria weirii did not use nitrate as a nitrogen source but grew well with ammonium or amino nitrogen (Table 1). Its behavior in culture was markedly similar to that of *Armillaria mellea* (Azevedo, 1963), another serious pathogen of tree roots. Moreover, the cell-free extracts of *P. weirii* completely lacked nitrate reductase activity (Table 2). The other method for determining nitrate reductase activity used nitrate as a hydrogen acceptor, the nitrate serving as a terminal oxidant for a reduced intermediate. Because inorganic nitrate provided the sole nitrogen source for all cultures, positive results indicate functioning systems for nitrate assimilation. This test also showed that *P. weirii* lacked nitrate reductase (Table 3). Lack of nitrate reductase in an organism under certain circumstances can be ecologically disadvantageous. The high nitrate content of the soils under the stand with red alder appears unfavorable to *P. weirii*: it cannot use nitrate nitrogen but antagonists such as *Streptomyces* spp. and other fungi can. Differences in nitrate reductase activity between the other fungi are difficult to interpret at this present state of investigation. If the higher nitrate reductase activity as determined by our procedure indicates greater

Table 1

Mean dry weight of mycelium of Poria weirii grown in synthetic media containing various sources of nitrogen*

Nitrogen (ppm)	Nitrogen sources		
	Potassium nitrate (mg)	Ammonium chloride (mg)	L-asparagine (mg)
10	2. 55	17. 75	20. 33
100	2. 00	32. 00	32. 48
1000	1. 28	37. 23	40. 08

*Means of four replicates

Table 2

Nitrate reductase activity of Neurospora crassa and Poria weirii

Reagent	Addition (ml)	(ml)	(ml)	(ml)
0.1M KNO_3	0.10	0.10	0.10	0.10
2.6×10^{-5}M FAD	0.05	0.05	0.05	0.05
2.0×10^{-3}M DPNH	0.04	0.04	0.04	0.04
0.2 M Pyrophosphate, pH 7.0	0.26	0.26	0.26	0.26
Enzyme extract	0.05	0.10	0.15	0.20
Distilled water	0.90	0.85	0.80	0.75
O. D. at 540 mμ for N. crassa	0.01	0.03	0.05	0.06
Nitrite formed for N. crassa	1×10^{-3} μ mole	2.5×10^{-3} μ mole	3.0×10^{-3} μ mole	4.5×10^{-3} μ mole
O. D. at 540 mμ for P. weirii	0	0	0	0
Nitrite formed for P. weirii	0	0	0	0

*Means of two replicates

Table 3

Nitrate reductase activity of cell-free extracts from duplicate cultures of selected fungi, expressed as μ moles of nitrite formed per milligram of dry mycelium

Fungus	Nitrite formed (μ moles x 10^{-2} per mg dry mycelium)	
	Per replicate	Mean
Fusarium avenaceum	0. 547 0. 564	0. 566
Fusarium oxysporum	0. 215 0. 224	0. 220
Gliocladium roseum	8. 110 8. 110	8. 110
Neurospora crassa	4. 600 4. 600	4. 600
Phytophthora cinnamomi	0. 476 0. 529	0. 503
Pythium debaryanum	1. 210 1. 210	1. 210
Poria weirii	0 0	0
Trichothecium roseum	0. 632 0. 469	0. 551
Verticillium albo-trum	0. 155 0. 155	0. 155

capability to assimilate nitrogen, then such differences could mean much in terms of competitive ability. Studies of inhibition of Fusarium oxysporum f. cubense by Agrobacterium radicola (Marshall and Alexander, 1960) hint that such might be the case. The major mechanism of suppression, in this instance, is reported to be competition for nitrate nitrogen rather than antifungal compounds produced by the bacterium. Relative efficiency in production of nitrate reductase could explain other changes that occur in populations of soil organisms when nitrate levels are changed. For example, Garrett (1940) reported that lowering soil nitrate levels by cropping practices decreases incidence of Ophiobolus graminis, cause of the "take-all disease" of wheat. Possibly O. graminis is not as efficient a producer of nitrate reductase as other nitrate-competing soil organisms. This is true with red alder which possesses the nitrate reducing capacity, presumably due to nitrate reductase. In one day 1 g of leaf tissue was able to form 0.196 μ mole nitrite (Table 4). However, no measurable activity was detected in Douglas-fir leaves. Beevers and Hageman (1963), Raghaven and Torrey (1964) and Rijien (1958, 1960) cited conclusive evidence that this enzyme is inductive in nature. Apparently, the lack of nitrate reducing capacity in Douglas-fir was due to the lack of its genetic ability to synthesize nitrate reductase (Townsend and Blatt, 1966). Therefore, manipulating populations of competing microorganisms through regulation of nitrate-nitrogen

Table 4

Reduction of nitrate to nitrite by plants and fungi

Species	Nitrite formed (μ mole/g of tissue/day)	Mean
Douglas-fir	0	
	0	0
	0	
Red alder	0.195	
	0.199	0.196
	0.193	
Rhizopogon occidentalis (Mycorrhizal)	0.252	
	0.267	0.257
	0.252	
Calvatia depressa (Non-mycorrhizal)	0.003	
	0.004	0.004
	0.004	

levels by red alder bears promising implications for biological control of pathogens, especially in soil. Hypothetically, relative efficiency of nitrate reducing capacity by different organisms is a key to predicting which organisms will succeed or fail with changes of available nitrate.

Microorganisms Antagonistic toward Poria weirii in Forest Soils

Soils under red alder and alder-conifer stands had a higher percentage of antagonistic fungi and Streptomyces than soil under the conifer stand (Table 5). From conifer soil, 16 percent of all fungi isolated were antagonistic toward P. weirii, whereas soils of alder and mixed stands yielded 40 percent and 39 percent antagonists respectively. In the alder-conifer soil, 40 percent of Streptomyces isolated were antagonistic toward P. weirii, 45 percent from the alder, and only 13 percent from the conifer soil. In another experiment, isolations at random from the soil dilution plates and testing their antagonistic action also revealed that the alder-conifer soil always had a higher percentage of antagonistic bacteria, Streptomyces, and fungi than the conifer stand soil, though the sampling was insufficient for statistical analysis.

The presence of large percentages of antagonists in soil under the mixed stand could help to minimize the incidence or severity of root-rot disease of conifers caused by P. weirii. Moreover, the

Table 5

Percentage distribution of microorganisms antagonistic against Poria weirii in the soils of alder, conifer, and mixed alder-conifer stands[1]

	Streptomyces			Fungi		
		Antagonists			Antagonists	
stand[2]	Total (10,000/g)	No. (10,000/g)	%	Total (1,000/g)	No. (1,000/g)	%
Alder-conifer	150	60	40	192.50	75	39
Red alder	160	72.5	45	34.75	13.75	40
Conifer	137.5	17.5	13	235	37.50	16

[1] Data are means of two replicates

[2] Soils collected in February 12, 1969.

high content of nitrate nitrogen under alder and the mixed stands provides a good nitrogen source for many of the antagonists. Thus, red alder in the mixed stand encourages microorganisms which compete with or antagonize P. weirii. Success in the use of antibiotic-producing organisms to suppress seed- and seedling-infecting pathogens has been achieved: the damping off of Scots-pine seedlings caused by Fusarium spp., has been controlled by treating the seeds with antibiotic forming bacteria (Krasilnikov and Raznitsina, as cited by Alexander, 1961), and Pythium infection of white mustard seeds has been controlled by dusting the seeds with spores of Trichoderma viride and other fungi (Wright, 1956).

Extracellular Phenoloxidases Produced by P. weirii

The cultural fluid collected from a three-week-old culture of P. weirii grown in synthetic medium was able to oxidize hydroquinone, catechol, and DL-dopa. However, it could not oxidize p-phenylenediamine, p-cresol, and tyrosine (Table 6). Certain phenoloxidases in wood-rotting fungi are related to lignin-decomposing ability (Etheridege, 1957; Käärik, 1965; Lindeberg, 1948; Schánĕl, 1967; Haider and Grabbe, 1967). Lignin in the presence of phenoloxidases absorbs oxygen and becomes more water soluble (Dion, 1952). Poria weirii is a lignin decomposer, producing a yellow ring rot in conifer wood; when grown on gallic or tannic acid media, it

Table 6

Color changes caused by cultural fluid collected from a three-week-old culture of Poria weirii, acting on phenolic substances

Treatments	Color changes
Cultural fluid + p-cresol	-[a]
Boiled cultural fluid + p-cresol	-
Cultural fluid + p-phenylenediamine	-
Boiled cultural + p-phenylenediamine	-
Cultural fluid + hydroquinone	+[b]
Boiled cultural fluid + hydroquinone	-
Cultural fluid + catechol	+
Boiled cultural fluid + catechol	-
Cultural fluid + DL-dopa (DL-dihydroxyphenylalanine)	+
Boiled cultural + DL-dopa	-
Cultural fluid + tyrosine	-
Boiled cultural fluid + tyrosine	-
Cultural fluid + sterile distilled water	-

[a]Color change negative

[b]Color change positive

produces a brownish diffusion zone (Nobles, 1948), indicating presence of phenoloxidase. However, this oxidative enzyme actually is comprised of two different enzymes: o-diphenol oxidase, which catalyzes the oxidation of catechol and other o-diphenols as DL-dopa, and p-diphenol oxidase which catalyzes the oxidation of diphenols of the hydroquinone type (Keilin and Mann, 1959). It has been established that phenoloxidases produced by white-rot fungi are able to damage the cell membrane components and decay ensues (Schánĕl, 1967). The studies by Lindeberg (1948) showed that a phenoloxidase regularly and abundantly is to be found in litter-decomposing basidiomycetes which attack lignin and cellulose, the two cell wall components in plants.

Phenoloxidases and Peroxidase in Red Alder and Douglas-fir

Red alder leaves and roots possess phenoloxidases which catalyze the oxidation of chlorogenic acid, catechol and DL-dopa. Chlorogenic acid appears the most reactive substrate. The extract from Douglas-fir leaves and roots showed no phenoloxidase activity for these substrates (Figures 1 to 3). Leaves of both red alder and Douglas-fir possess peroxidase, but the former showed much higher activity than the latter (Figure 4). Poria weirii can penetrate the wood of both Douglas-fir and red alder, but in the case of alder the bark generally must be injured first and even then growth of the

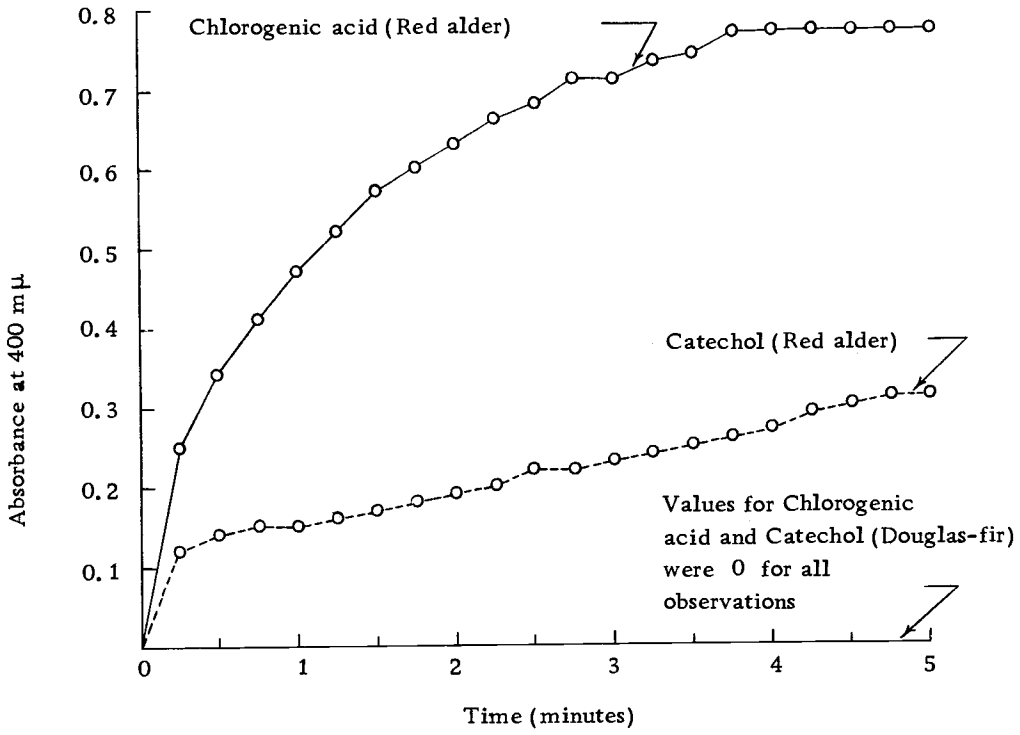


Figure 1. Phenoloxidase activity of leaf extracts obtained from red alder and Douglas-fir. Assay: substrates, chlorogenic acid and catechol 1 x 10^{-3} M, 2.9 ml; enzyme extract, 0.1 ml.

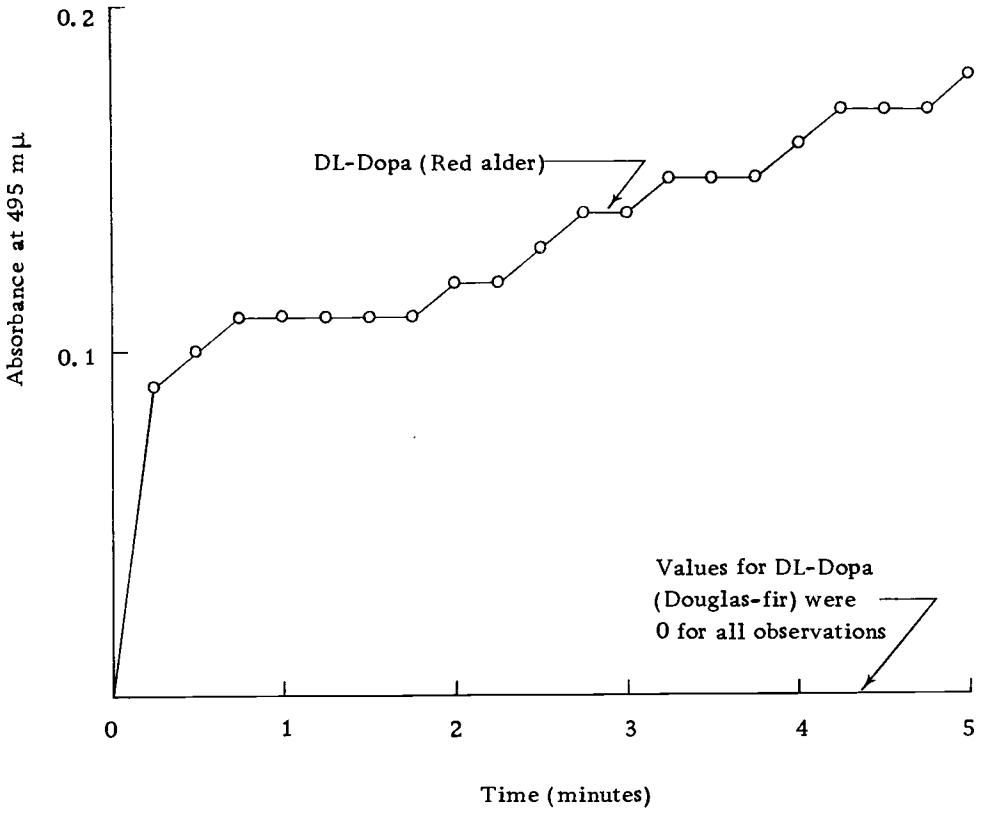


Figure 2. Phenoloxidase activity of leaf extracts from red alder and Douglas-fir. Assay: substrate, DL-dopa, 1×10^{-3}M, 2.9 ml; enzyme extract, 0.1 ml.

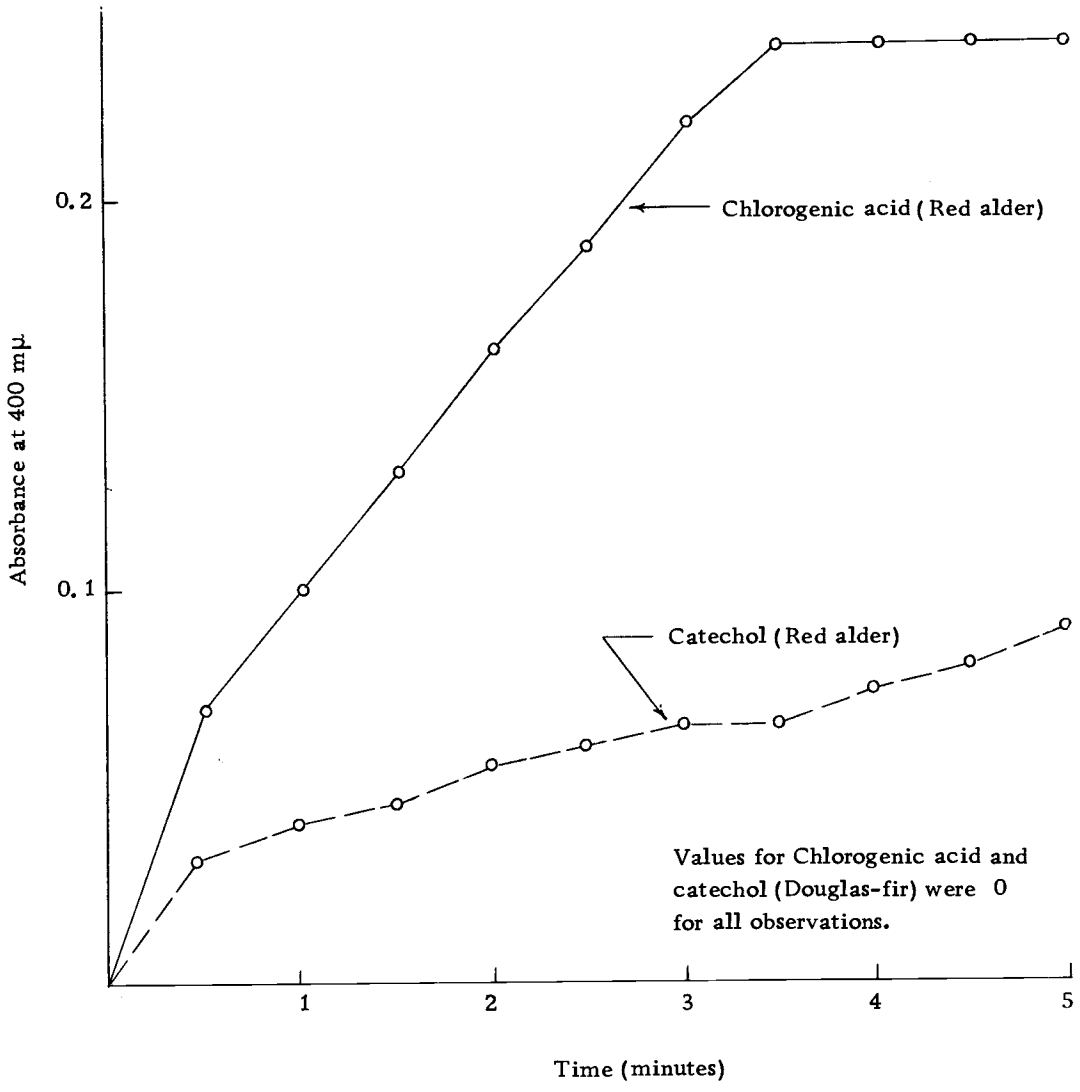


Figure 3. Phenoloxidase activity of root extracts obtained from red alder and Douglas-fir. Assay: substrates, chlorogenic acid and catechol 1 x 10^{-3} M, 2.9 ml; enzyme extract, 0.1 ml.

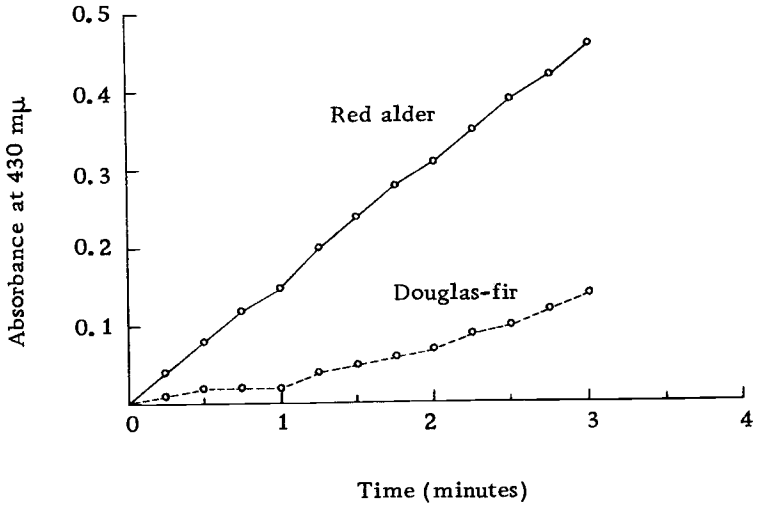


Figure 4. Peroxidase activity of leaf extracts obtained from red alder and Douglas-fir. Assay: Pyrogallol, 0. 2 M, 0. 1 ml; McIlvaine's buffer, pH 7. 0, 1. 0 ml; H_2O_2, 0. 01 M, 0. 5 ml; and redistilled water, 0. 9 ml.

fungus is restricted to a small zone around the point of entry (Wallis and Reynolds, 1965). Heat labile compounds evidently account for resistance of alder wood to growth of the fungus: P. weirii will grow well in the autoclaved wood (Wallis and Reynolds, 1962), but not in previously unheated wood (G. W. Wallis, personal communication).

The heat labile compounds prominently involved in alder's resistance to P. weirii probably included phenoloxidases that catalyze oxidation of chlorogenic acid and other phenols in the tissue into fungitoxic end-products (Hegnauer, 1964; Rubin and Artsikhovskaya, 1964; Hare, 1966). The products of phenol oxidation would then accumulate in infected cells and polymerize, forming dark, resin-like substances which impregnate the tissue and form a barrier to further spread of the mycelium (Rubin and Artsikhovskaya, 1963 and 1964). It is also possible that these or other oxidized phenols in the infected tissue inactivate or precipitate the extracellular phenoloxidases produced by P. weirii, thereby preventing lignin degradation by the fungus (Byrde et al., 1960; Loomis and Battaile, 1966). Chlorogenic acid, caffeic acid, and protocatechuic acid have been reported to be present in red alder (Hegnauer, 1962 and 1964), and Gordon and Paleg (1961) reported that these phenolic compounds can react with tryptophane to form the auxin indoleacetic acid (IAA), the action being catalyzed by the phenoloxidase enzymes of plants. They also suggested that the tryptophan-phenoloxidase-phenol system might be

activated by wounding of tissues; IAA produced could induce the hypertrophy and hyperplasia of associated callus tissue. Subsequent studies by Beckman et al. (1962) have demonstrated that IAA will induce tylosis in roots of Fusarium-wilt resistant and susceptible bananas. Tylosis occurs rapidly enough in the resistant banana to limit spread of Fusarium and confer resistance; whereas it is sufficiently delayed in the susceptible variety to permit systemic spread and disease development.

Red alder has high peroxidase activity, which might increase considerably in hypersensitive red alder reaction-tissue during invasion by P. weirii hyphae (Rubin and Artsikhovskaya, 1963 and 1964). Its role in resistance of tree roots to pathogenic infection, however, is unexplored. Fehrmann and Dimond (1967) have postulated that peroxidase activity is one attribute of host tissue contributing to environmental inhospitality for a fungus.

To recapitulate these interpretations, the phenolic compounds and phenoloxidases present in red alder but not detected in Douglas-fir can be hypothesized as a primary biochemical source of alder's resistance to P. weirii by (1) resulting in oxidation of phenolic compounds into fungitoxins at the infection site, and (2) destroying the lignin decomposing ability of P. weirii by inactivating its extracellular enzymes. The relatively high peroxidase activity may further contribute to resistance against the fungus. Because P. weirii

spreads by growth along infected root systems (Wallis and Reynolds, 1965), the mere presence of resistant alder trees in a stand reduces spread by reducing the chances for root contacts between susceptible trees.

Effect of pH and Temperature on Growth of P. weirii in vitro

Statistical significance of relationships between final weights or diameters of colonies and treatments was tested by regression analysis. Though the need to use different incubators for temperature treatments precluded randomization, the possibility of extraneous factors affecting fungus growth in this kind of experiment is remote, so regression analysis can be assumed to be valid.

Mean dry weight of P. weirii increased with pH at an accelerating rate between pH 3.0 and 6.0. The fall from maximum growth at pH 6.0 to no growth at pH 6.5 and above was abrupt (Figure 5). At time of harvest, the pH of each medium generally was higher than at the outset except for those starting at pH 5.5 or higher (Table 7). Mean dry weight of P. weirii colonies increased with temperature from 5°C to a maximum at 20° then fell off abruptly at temperatures above to no growth at 30° or higher (Figure 6). The resulting "S" curve proved highly significant in regression analysis. Identical trends in diameter growth on malt agar were also highly significant (Table 8).

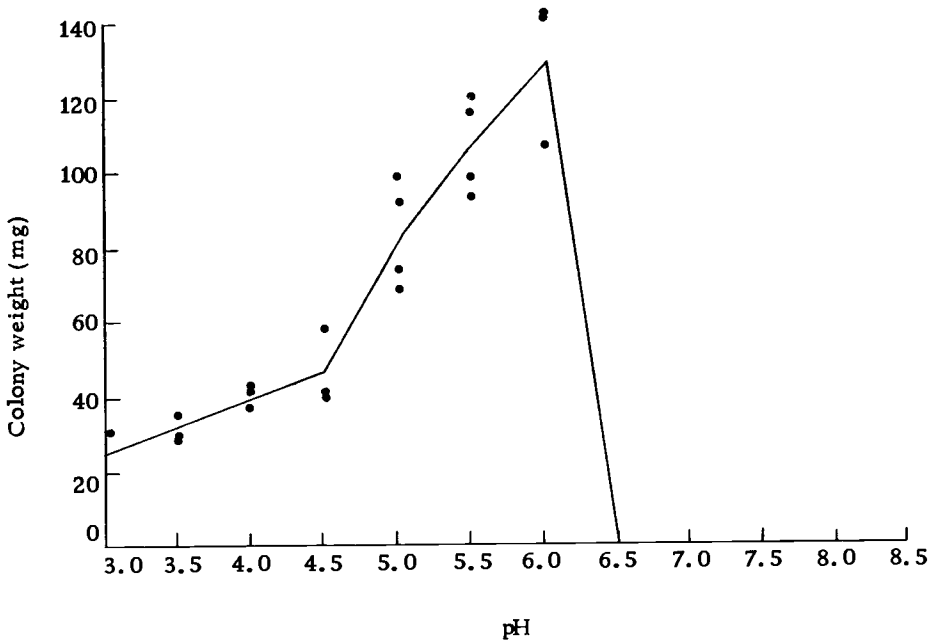


Figure 5. Weights of colonies of Poria weirii grown for 30 days in media with different beginning pH values (four replicates at each pH).

Table 7

Dry weight of mycelium of Poria weirii grown 30 days in a synthetic medium at various pH values

Initial pH	Mean colony weight* (mg)	Final pH
3.0	24	4.3
3.5	31	5.0
4.0	40	5.0
4.5	46	4.6
5.0	84	5.7
5.5	107	5.3
6.0	130	5.7
6.5	0	6.5
7.0	0	7.0
7.5	0	7.4

*Means of four replicates

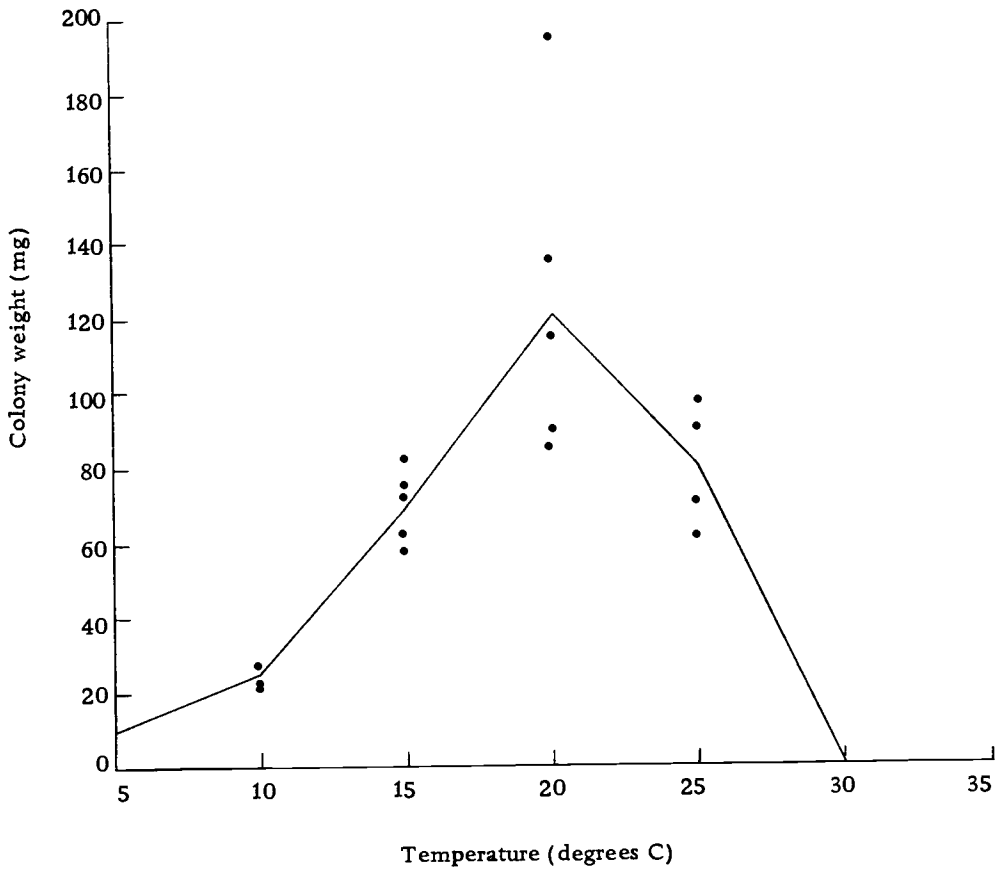


Figure 6. Weights of colonies of Poria weirii grown for 30 days in synthetic medium at different temperatures (five replicates at each temperature).

Table 8

Radial growth of mycelium of Poria weirii on malt agar at various temperatures

Days	Diameter of colony* (mm)						
	5° C	10° C	15° C	20° C	25° C	30° C	35° C
3	0	0	13. 20	21. 60	21. 80	0	0
6	0	10. 00	24. 00	38. 20	36. 00	0	0
9	0	16. 80	38. 80	62. 60	48. 60	0	0
12	0	25. 00	55. 40	84. 20	63. 20	0	0

*The data were based on means of five replicates at each temperature.

The purpose of this study was to establish pH and temperature optima for a single isolate to facilitate other research with that isolate. Though the range of variation within this species in respect to these optima remains to be defined, we can reasonably anticipate that isolates of P. weirii will vary from each other in pH and temperature optima for growth, as strains or geographical isolates of a given species may respond differently (Cowling and Kelman, 1964; Etheridge, 1955; Humphrey and Siggers, 1933). The results of this study also indicate that the lower pH associated with red alder may not be an important factor for inhibiting invasion of P. weirii. It may be, however, a major factor affecting the availability of soil nutrients, which in turn influence the prevalence of certain antagonistic soil microflora (Kaufman and Williams, 1964).

Inhibition of Poria weirii and Fomes annosus by Linoleic Acid

An inverse cubic relationship between weight of P. weirii and increasing concentration of linoleic acid salt proved highly significant when the whole range of data were analyzed. However, this mathematical relationship proved untenable from a biological standpoint, because the curve leveled off with increase in linoleic acid salt concentrations higher than 2.0 mg/ml. Because growth and variation were uniformly minimal at 1.0 mg or higher concentrations, a second polynomial regression was run using only the data from

concentrations in which P. weirii demonstrably grew (0. 0 to 0. 75 mg/ml).

In this second analysis, the quadratic and cubic relationships were nonsignificant. The highly significant inverse linear regression was expressed as mg of P. weirii = 2. 75 - 1. 603 (mg/ml linoleic acid salt) (Figure 7). In other words, through the 0. 75 mg/ml concentration the 22-day growth of P. weirii was reduced from that of the control by a factor of 1. 6 x each mg increment of linoleic acid salt in the solution. Growth fell to practically nothing when the concentration was increased from 0. 75 to 1. 0 mg/ml, and was identically low at all higher concentrations.

In the case of F. annosus, the fungus grew moderately well at all concentrations tested. However, an increase of inhibition occurred with each increase of concentration of linoleic acid salt (Figure 8), as expressed by the following highly significant inverse linear regression:

mg of F. annosus = 1. 496 - . 1494 (mg/ml linoleic acid salt).

This result suggests that linoleic acid is an important factor in the resistance of red alder to infection by P. weirii (Wallis, 1968) and plays a role in reducing longevity of this fungus in soil under alder. Such a mechanism of alder's antifungal activity could be important for development of concepts in biological control of root

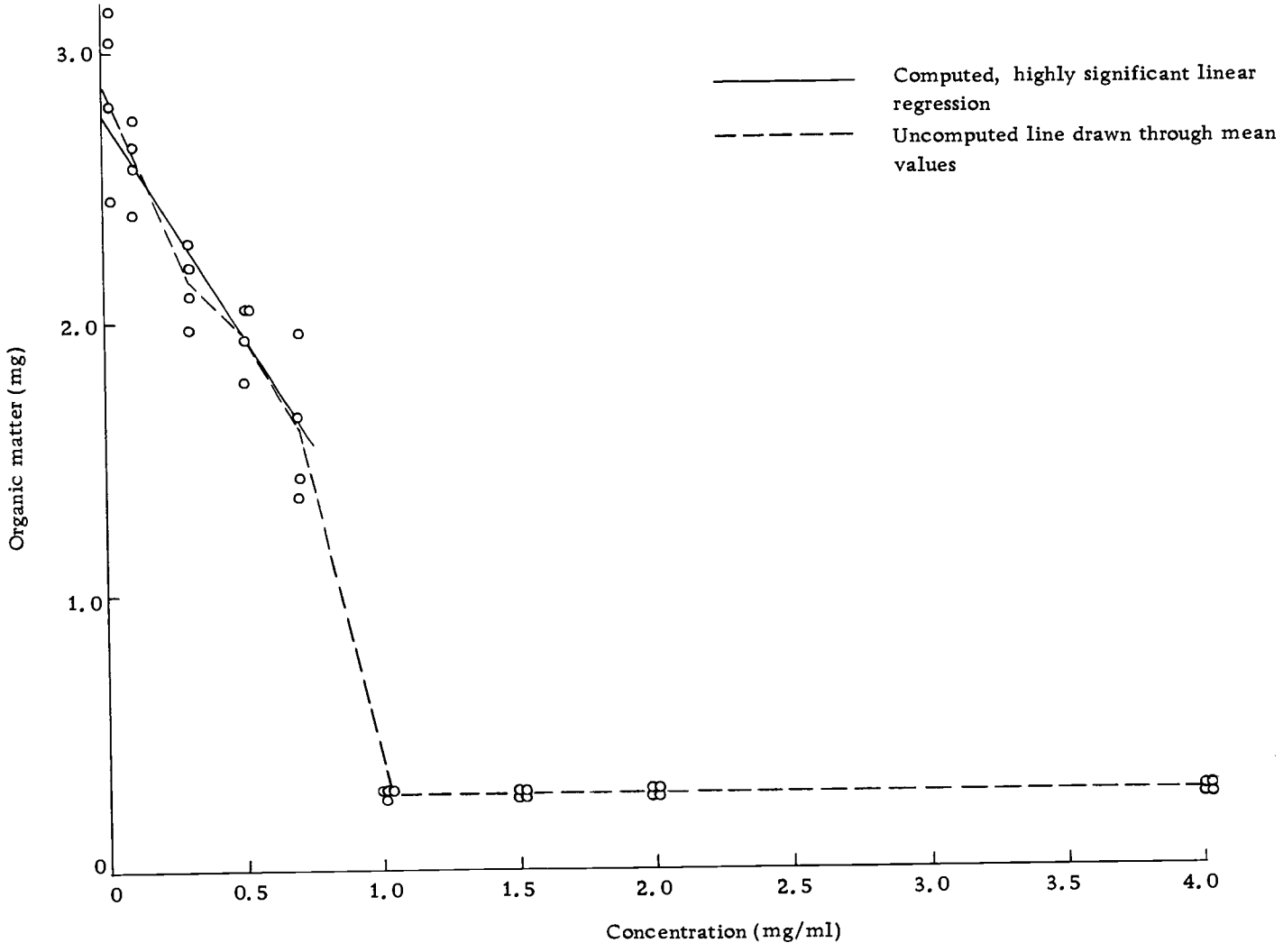


Figure 7. Amount of organic matter in mycelium of Poria weirii grown for 22 days in media with different concentrations of linoleic acid.

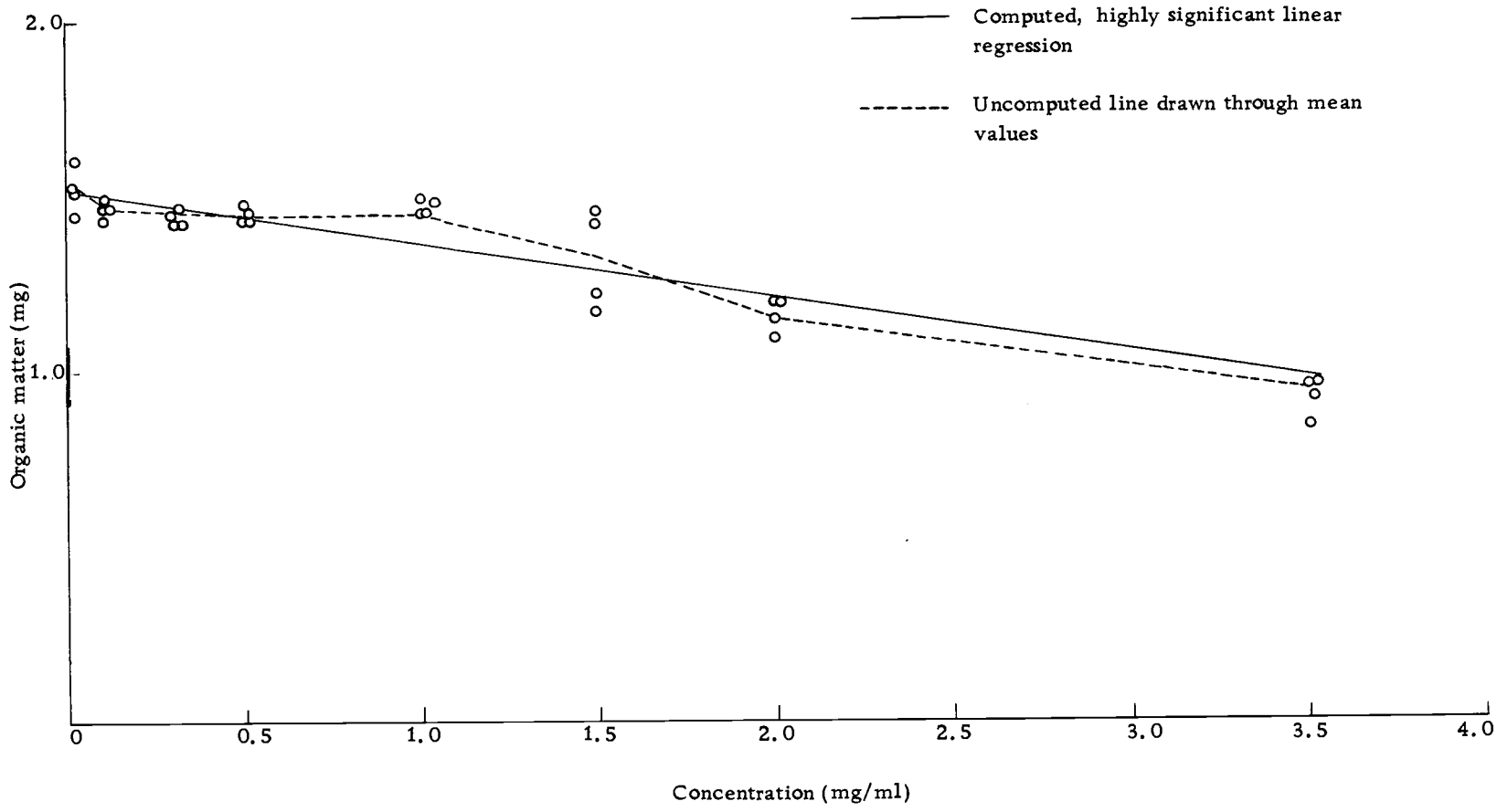


Figure 8. Amount of organic matter in mycelium of Fomes annosus grown for 17 days in media with different concentrations of linoleic acid.

diseases in forests. A similarly strong inhibition of F. annosus in nature seems less likely on the basis of our data.

Effects of Phenolic and Related Compounds on Growth of Poria weirii

Data were subjected to analysis of variance using the Scheffé test for significance of differences between means at the 95 percent confidence level (Snedecor and Cochran, 1967). Certain treatment means were excluded from the analysis where the assumption of equal variences did not hold. Mean weights for all treatments are shown in Table 9.

Interpretation of the effects of the compounds on growth of P. weirii must be tempered by the possibility of their transformation in reaction with other components of the medium or enzyme activity of the fungus. However, for convenience in presenting results, the data refer to the named compounds as originally added. Addition of a greater volume of ethanol to flasks of the 2.0 mM concentration treatments produced apparent, though not statistically significant, stimulation of both isolates (Table 9). Since this effect could influence fungal growth, mean mycelial weights are statistically compared between the control and compounds at a given concentration but not between concentrations of a given phenolic compound.

At the 2.0 mM level, both isolates of P. weirii were strongly

Table 9

Mean mycelial weights of two isolates of Poria weirii (T-55 and T-124)[1] grown on media to which phenolic and related compounds were added at two concentrations

Compound	Mean weight[2] Phenolic concentration				Mean final pH of medium Phenolic concentration			
	0.5 mM		2.0 mM		0.5 mM		2.0 mM	
	T-55	T-124	T-55	T-124	T-55	T-124	T-55	T-124
Control[3]	165[5]	155	264	215	5.5	6.0	3.9	4.1
Catechols								
o-catechol	177	137	7	14	6.1	5.4	5.8	5.8
hydroquinone	132	149	216	104	5.4	4.8	4.0	4.1
phloroglucinol	92	128	130	224	6.0	5.2	4.8	3.7
Benzoic acids								
Benzoic acid	227	144	0	0	6.2	4.6	5.4	5.4
salicylic acid	142	141	0	0	5.2	4.1	5.4	5.4
p-hydroxybenzoic acid	251	169	338	193	6.0	4.2	3.4	4.0
protocatechuic acid	199	177	334	302[4]	6.1	5.0	4.2	3.9
gentisic acid	108	167	136	271	6.1	4.5	6.5	4.5
gallic acid	150	182	217	253	5.8	4.5	6.3	4.3
vanillic acid	120	141	161	30	6.4	4.6	4.6	4.5
syringic acid	95	119	113	245	6.1	4.0	6.2	3.9
Cinnamic acids								
o-coumaric acid	188	140	4	20	6.0	5.8	4.3	4.6
p-coumaric acid	175	220	265	196	6.4	4.7	5.0	4.4
caffeic acid	110	158	144	231	6.3	4.7	6.5	4.3
ferulic acid	108	136	0	0	6.2	4.5	5.4	5.4
Phenylacetic acids								
phenylacetic acid	92	132	0	0	6.2	5.0	5.4	5.4
o-hydroxyphenylacetic acid	131	160	193	166	6.5	5.5	5.0	3.8
p-hydroxyphenylacetic acid	182	146	272	235	6.2	4.6	6.0	4.0
3, 4-dihydroxyphenylacetic acid	106	131	77	205	6.3	5.4	4.2	3.9
Coumarins								
coumarin	22	48	6	14	5.0	4.6	5.5	5.2
4-hydroxycoumarin	95	34	8	0	4.4	4.4	4.4	5.4
7-hydroxycoumarin	52	63	9	12	5.4	4.1	5.7	5.6
Others								
chlorogenic acid	113	170	132	223	6.7	4.1	6.3	4.2
D-catechin	104	198	148	327[4]	6.1	5.8	5.5	3.6
phloridizin	171	154	224	220	5.8	4.9	6.8	3.7

[1] Incubated at 20°C for 29 days and 22 days, respectively.

[2] Underlined means are significantly lower than the control for the same column at the 95% confidence level; in case of unequal variance, inhibition was obvious.

[3] Equivalent amounts of 50% ethanol added as for ethanolic solutions of test compounds.

[4] Significantly higher than control for same column at the 95% confidence level.

[5] All values are given in mg. for Phenolic concentration mean weight.

inhibited by nine compounds: o-catechol, benzoic acid, salicylic acid, o-coumaric acid, ferulic acid, phenylacetic acid, coumarin, 4-hydroxycoumarin, and 7-hydroxycoumarin. The mean mycelial weights were so exceptionally low in these treatments that they were excluded from the statistical analysis to avoid violating the assumption of equal variances. For convenience of presentation in Table 9, these means are shown as significantly lower than controls in the same way as means that were submitted to statistical analysis.

None of the other compounds significantly affected both isolates of P. weirii at the 2.0 mM concentration. Vanillic acid significantly inhibited T-124 and appeared to depress growth of T-55. D-catechin inhibited T-55 but stimulated T-124, both at a significant level. Gentisic acid produced a similar response, but only the inhibition of T-55 was statistically significant. Protocatechuic acid significantly stimulated T-124 with a similar but not significant trend for T-55.

The only significant effect at the 0.5 mM level was rather consistent suppression of both isolates by the three coumarins, which were strongly inhibitory at the 2.0 mM level. No consistent relationships were apparent between growth of the fungi and final pH of the medium. During the course of the experiment, in most treatments pH of the medium with isolate T-124 was lowered. With T-55, however, final pH of many treatments was substantially higher than at the beginning.

Those compounds which showed strong inhibitory effects on growth of both isolates of P. weirii at 2.0 mM concentration, such as phenylacetic acid, salicylic acid, and coumarin, also inhibited the endogenous respiration of P. weirii T-124 (Figure 9), though the inhibition by coumarin was not very significant. Chlorogenic acid and gallic acid which showed stimulatory effects on growth of T-124 also stimulated the endogenous respiration of this isolate.

The results of this study generally parallel those of Christie (1965) for Phytophthora cactorum (Leb. and Cohn) Schroet. and P. parasitica Dast. The two studies diverge in that Christie found strong inhibition of both Phytophthora species by p-hydroxybenzoic acid, p-coumaric acid, and o-hydroxyphenylacetic acid, all of which were noneffective or stimulatory to P. weirii.

Structural formulae of all compounds tested except the paradoxical D-catechin are grouped in Figure 9 by their relative effect on P. weirii. Although this grouping is obviously an oversimplification, it does facilitate detection of possible relationships between structure and effect of compounds. Clearly, the double-ring coumarins stand out as the strongest inhibitors. Within the catechols, benzoic acids, and cinnamic acids, some features are generally associated with increased inhibition: (1) lack of a hydroxyl (-OH) group, or (2) only two positions on the benzene ring occupied, and these in the ortho position, or (3) addition of methoxyl(H_3CO-)

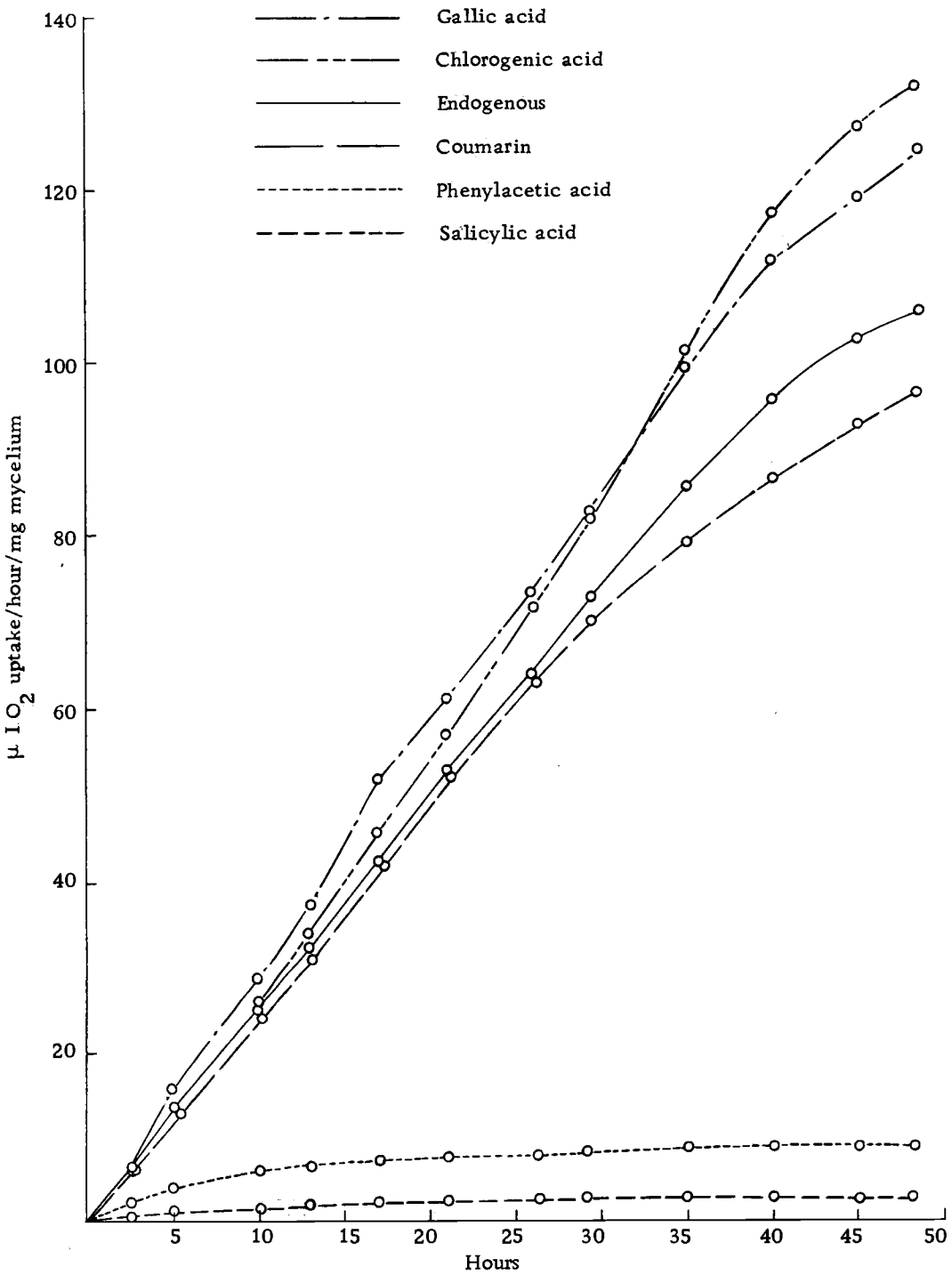


Figure 9. Effect of phenolic compounds on endogenous respiration of P. weirii T-124.

groups. The phenylacetic acids conformed to the first of these generalities, not to the second, and had no methoxyl groups to test the third. In general, these observations support the conclusions of Christie about the relationship of structure to inhibition of Phytophthora species, but too little is yet known on this topic to permit broad conclusions.

Of the compounds noted in the compilation of Hegnauer (1964) and the work of Tomaszewski (1960) as present in alder species, only caffeic acid, chlorogenic acid, and gentisic acid proved inhibitory to P. weirii, and only to one of the two isolates at the 2.0 mM concentration. Indeed, protocatechuic acid, p-hydroxybenzoic acid, p-coumaric acid, and gallic acid were noneffective or stimulatory. Judging from results of these tests, none of these compounds in themselves seem important in resistance of red alder to P. weirii in terms of direct toxicity or oxidation to toxic compounds as catalyzed by fungus-secreted enzymes. They cannot be discounted as resistance factors, however, because red alder itself may produce the enzymes that catalyze their oxidation into fungitoxins. Multiplication of fungitoxic effects through synergisms between two or more compounds is also possible.

Total Phenolics in Plants and Soils

The content of total phenolics (tannin content) varied widely in the plants from different tissues (Table 10). Root and litter of red

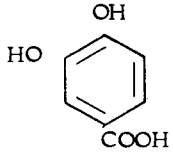
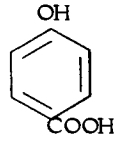
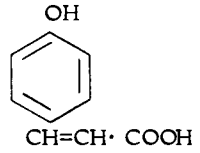
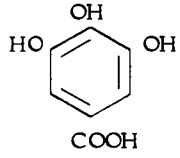
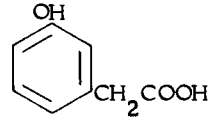
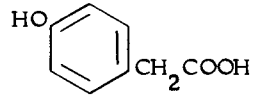
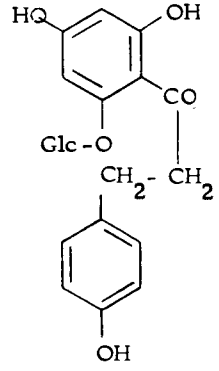
Relative effect on Poria weirii	Catechols	Benzoic acids	Cinnamic acids	Phenylacetic acids	Coumarins	Others
Stimulatory		 Protocatechuic acid				
Weakly stimulatory		 p-hydroxybenzoic acid	 p-coumaric acid			
Noneffective		 Gallic acid		 o-hydroxyphenyl acetic acid  p-hydroxyphenyl acetic acid		 Phloridzin

Figure 10. Structural formulae of 24 phenolic compounds, grouped by their relative effect on Poria weirii in vitro.

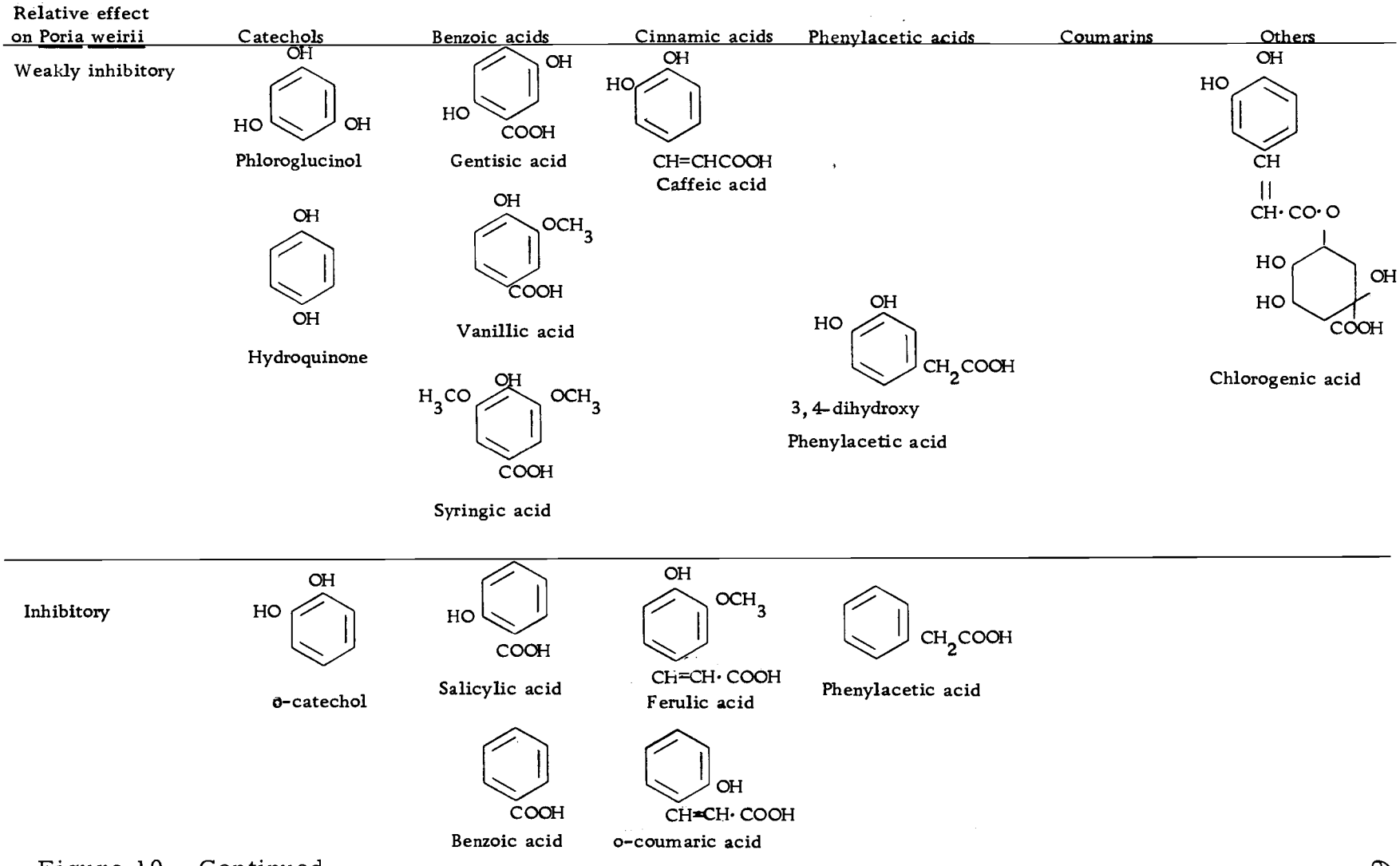


Figure 10. Continued.

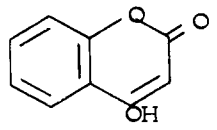
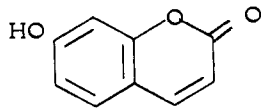
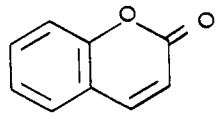
Relative effect on *Poria weirii*	Catechols	Benzoic acids	Cinnamic acids	Phenylacetic acids	Coumarins	Others
Strongly inhibitory					 4-hydroxycoumarin	
					 7-hydroxycoumarin	
					 Coumarin	

Figure 10. Continued.

Table 10

Total phenolics in plants and forest soils

	Red alder	Douglas-fir	Alder-conifer mixed stand
	(Total phenolics, tannic acid equivalent mg/g dry weight)*		
Roots	51.08	27.16	-
Litter	0.16	0.08	-
Fresh leaves	105.48	13.71	-
Soils	0.86	0.48	0.97

*Mean of three replicates

alder contained two times as much tannins as those of Douglas-fir; the fresh leaves of the former contained about eight times as much as that of the latter. Fresh red alder leaves had two times as much total phenolics as the roots, whereas the reverse was true with Douglas-fir. Soil under the alder-conifer interplanted stand had higher tannin content than the soil under conifer or under alder, and about two times as much as in the Douglas-fir. Red alder soil had higher total phenolics than the Douglas-fir soil. The present method of tannin determination involved the use of crude tannin extract. No means were used for isolation and fractionation of total phenolic constituents and the method did not distinguish between different types of tannins and may have included non-tannin compounds in the extracts.

There is very little information in the literature concerning the relative toxicity of the different types of tannins. Extensive experiments by Cook and Taubenhaus (1911) demonstrated the toxic effect of a variety of tannins on 18 species of saprophytic and pathogenic fungi with regard to *in vitro* spore germination, mycelial growth, and sporulation. Lewis and Papavizas (1967) demonstrated that tannins were able to inhibit spore germination and mycelial growth of *Fusarium solani* (Mart.) Appel and Wr. f. *phaseoli* (Burk.) Synd. and Hans., and *Verticillium albo-atrum* Reinke and Bert., important soil-borne plant pathogens.

High molecular weight tannins, formed in situ by oxidative condensation of monomeric precursors, are probably of primary significant in plant resistance to root diseases, and lend support to the theory of their general involvement in disease resistance mechanisms. Inactivation of enzymes and other biologically active proteins by oxidized phenols have been reported extensively (Hoffmann-Ostenhof, 1963; Wood, 1961). In some plant diseases, inactivation of extracellular enzymes of pathogens by oxidized phenols of plants is considered to be a major defense mechanism of the host (Byrde, 1963). Wounding of the resistant root tissue may produce oxidation products which might then inactivate the enzyme system of the pathogen and hinder its further development in the host.

Although not established with presented data, P. weirii was not able to grow on malt agar containing 0.5 percent tannic acid (Figure 11); the tannic acid was oxidized to brownish products which then retarded the growth of P. weirii. Poria weirii in this case became self-inhibited.

Another explanation of the toxicity of tannins is that it may be caused by their phenolic constituents which are relased by the enzymatic activities of the parasite (Newton and Anderson, 1929; Offord, 1940). The tannase of a certain fungus decomposes only a certain type of tannin, as shown by the work of Fuller and Nierenstein (1944). Freudenberg and Walpuski (as cited by

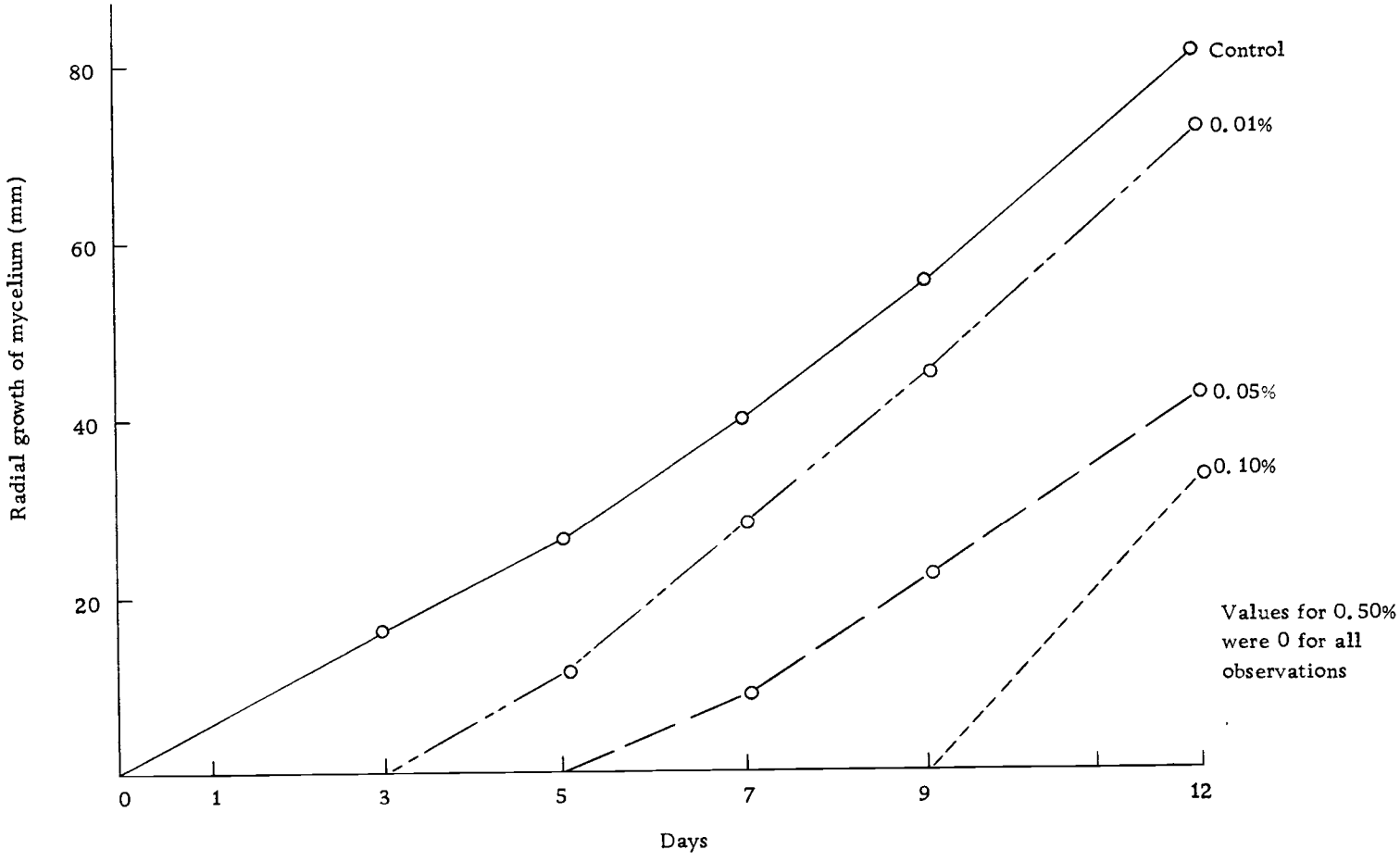


Figure 11. Effect of tannic acid on mycelial growth of Poria weirii on malt agar.

Nienstead, 1953) studied the decomposition of tannins of European chestnut by Aspergillus-tannase and obtained traces of gallic acid, ellagic acid and sugar. No information is available concerning what the tannase decomposition of the tannins of red alder and Douglas-fir may yield, or the type of enzymes produced by P. weirii. Until additional information is available the following is suggested as a working hypothesis explaining the role of the tannin quality in resistance to P. weirii: the tannase complex of P. weirii decomposes red alder tannins and in doing so produces phenolic compounds which are toxic to the fungus, whereas the decomposition of Douglas-fir tannins produces other phenolics which are noneffective or stimulatory to the fungus. Thus, the red alder in an interplanted stand may provide physical and chemical barriers for Douglas-fir from attack by P. weirii.

Phenolic Compounds in Plants and Forest Soils

Fluorescence under ultraviolet light (350 mμ) before and after spraying with 2 N NaOH, color reactions with diazotized p-nitroaniline, and Rf values of the spots indicated ferulic acid and vanillic acid in red alder roots, whereas roots of Douglas-fir contained only vanillic acid (Table 11; Figures 12 and 13). Because the separation of compounds and Rf values are strongly influenced by moisture content of the thin layer plate and solvent system to

Table 11

Phenolic compounds identified in plants and forest soils

Compounds	Rf	U. V. light (350 mμ) Before NaOH	U. V. light (350 mμ) After NaOH	Color reaction with diazotized p-nitroaniline	Red alder	Douglas-fir
Ferulic acid	0. 85	Bluish violet	Violet blue	Grayish violet	Root	-*
Vanillic acid	0. 81	Invisible	Invisible	Grayish rose	Root, litter, fresh leaves, soil, mixed stand soil	Root, litter, soil (trace)
Caffeic acid	0. 07	Pale blue	Light Green	Grayish orange	Fresh leaves	-

*- indicates absence.

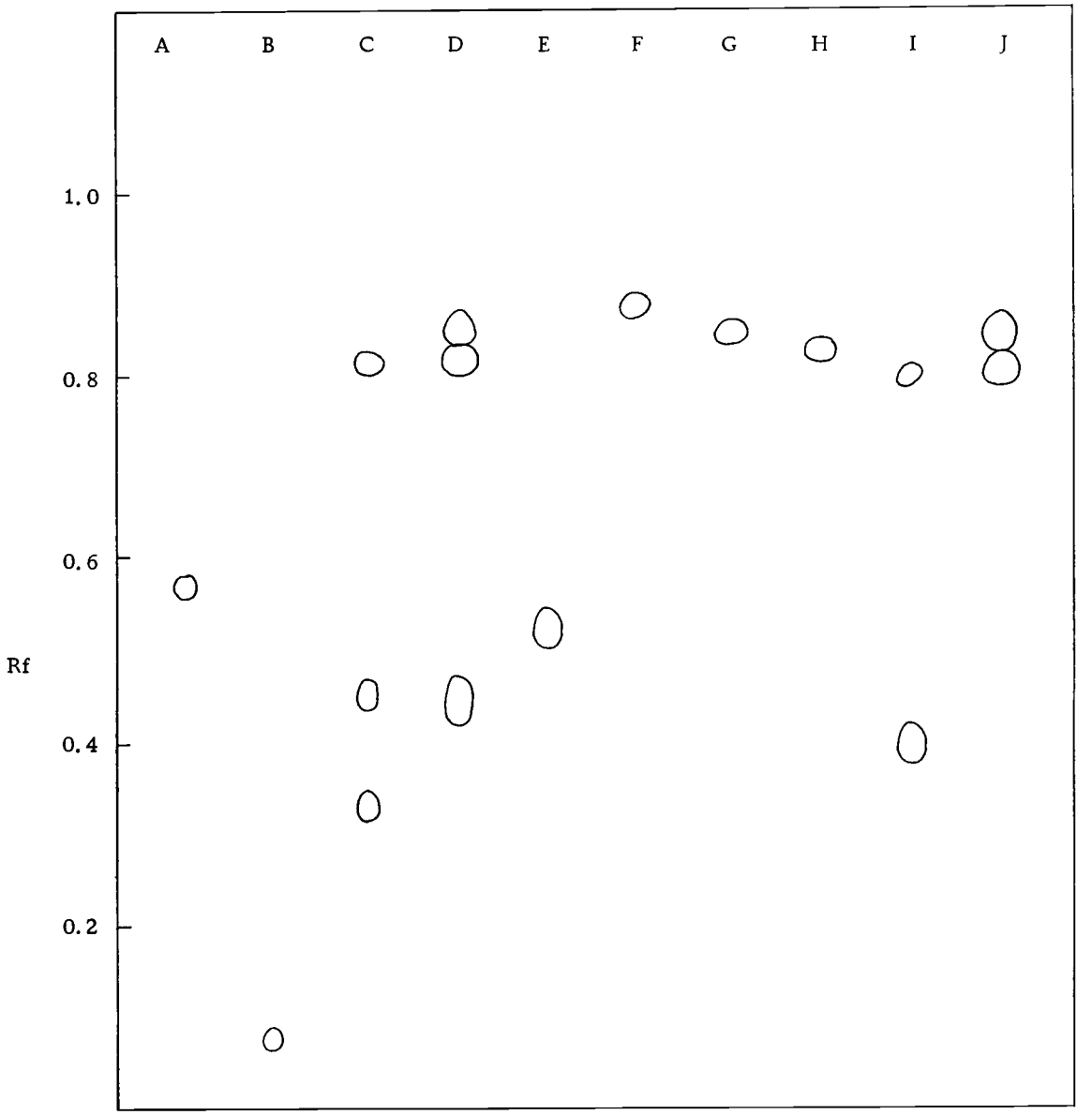


Figure 12. Chromatographic pattern of phenolic compounds from root extracts of Douglas-fir and red alder: A, 7-hydoxy-coumarin; B, caffeic acid; C, Douglas-fir (alkali hydrolysates); D, red alder (alkali hydrolysates): E, o-coumaric acid; F, salicylic acid; G, ferulic acid; H, Scopoletin; I, Douglas-fir (acid hydrolysates); J, red alder (acid hydrolysates). Thin-layer, steamed cellulose-silica gel G; solvent, chloroform-acetic acid-water (8:1:1).

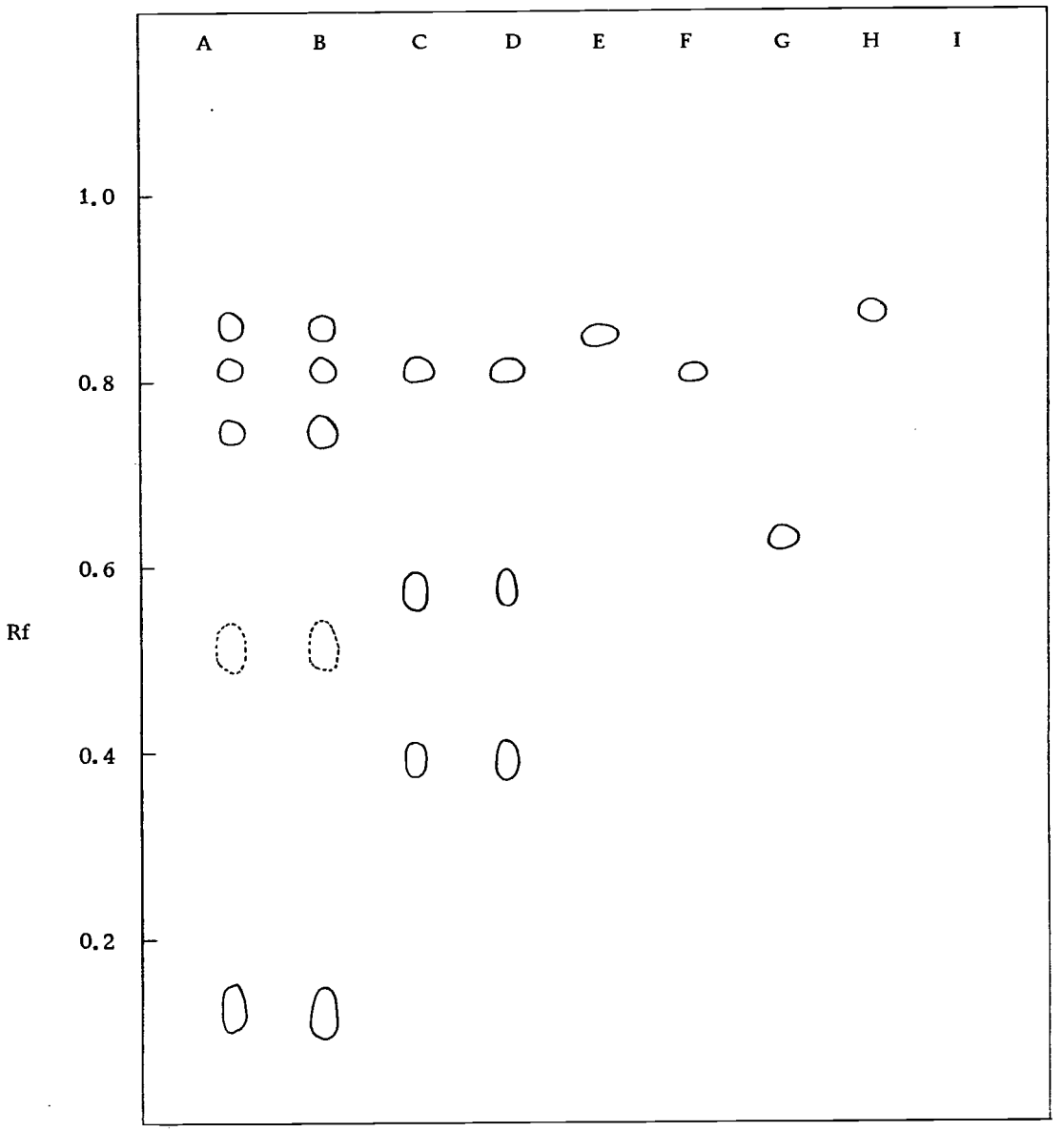


Figure 13. Chromatographic pattern of phenolic compounds from root extracts of Douglas-fir and red alder: A, B, red alder (acid hydrolysates); C, D, Douglas-fir (acid hydrolysates); E, ferulic acid; F, vanillic acid; G, 7-hydroxycoumarin; H, salicylic acid; I, chlorogenic acid, invisible. Thin-layer, steamed cellulose-silica gel G; solvent, chloroform-acetic acid-water (8:1:1). Faint spots are outlined with dots.

develop the chromatogram (Sumere et al. 1965, Figures 12 to 14), the Rf values of developed compounds were compared with the authentic compounds on the same plate. Vanillic acid was also detected in litter of both red alder and Douglas-fir, and in soils of red alder, conifer and mixed alder-conifer stands. However, the concentration in the mixed stand soil was highest and soil of the conifer stand contained only trace amounts. Vanillic acid and caffeic acid were detected in fresh leaves of red alder. No attempts were made to identify these compounds in the fresh leaves of Douglas-fir, as the separative resolution of the methods used for red alder extracts was too poor for Douglas-fir. There were several unknown compounds in red alder extracts; their respective Rf values were 0. 22, 0. 45, 0. 51, and 0. 74. In Douglas-fir extracts, there were unknown compounds with Rf values 0. 33, 0. 38, 0. 45, and 0. 57.

The literature on phenolic compounds of Douglas-fir and red alder tissues is scant, but some leads on compounds of possible importance in resistance to Poria weirii appear in the compilation of Hegnaur (1962, 1964), and the work of Kurth (1953), Hergert (1960) and Goldschmid and Hergert (1961). Compounds found in leaves of red alder but not reported from Douglas-fir are chlorogenic acid, caffeic acid, protocatechuic acid, and gallic acid. Bark or wood of Douglas-fir and western hemlock (Tsuga heterophylla (Raf.) Sarg.) contain protocatechuic acid, phloroglucinol, ferulic acid, vanillic acid, and D-catechin

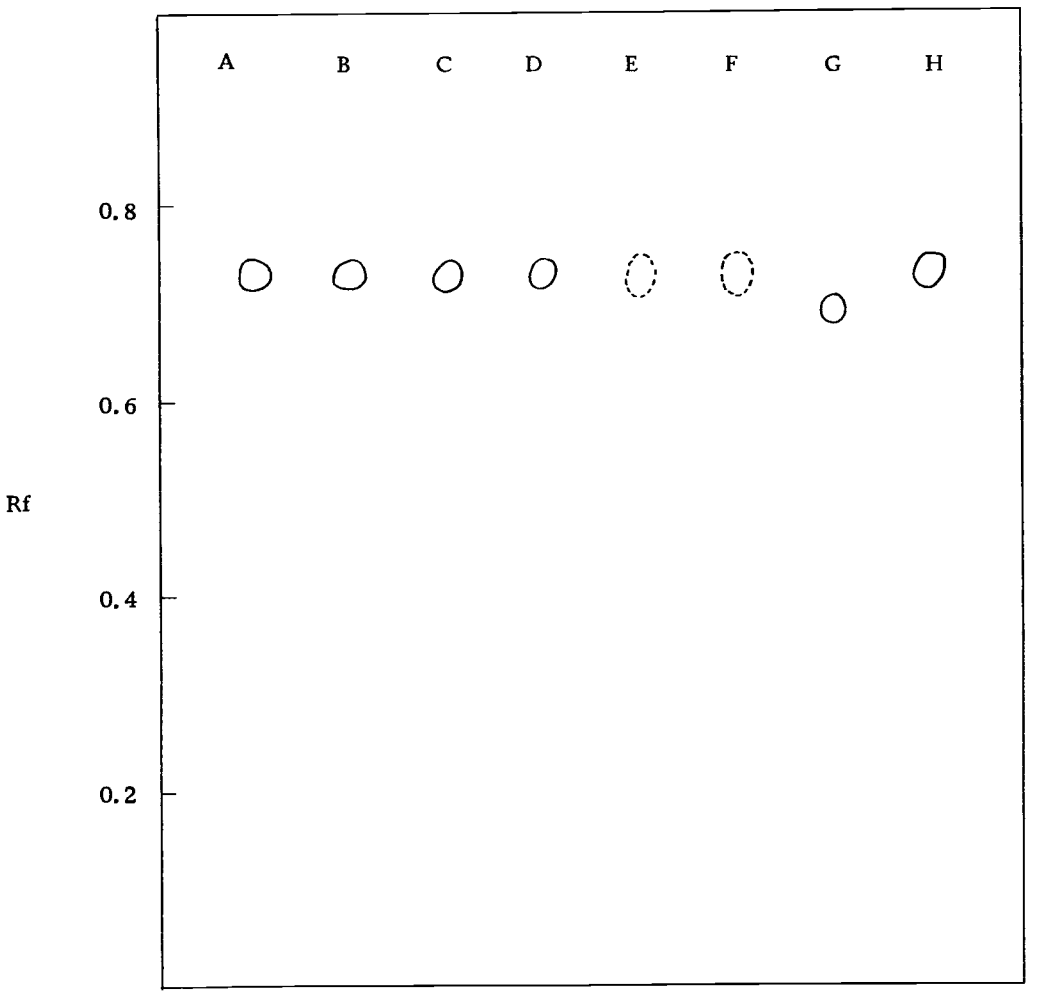


Figure 14. Chromatographic pattern of phenolic compounds from soils of Douglas-fir, red alder, and alder-conifer mixed stands: A, B, mixed stand; C, D, alder stand; E. F, Douglas-fir stand; G, ferulic acid; H, Vanillic acid. Thin-layer, steamed cellulose-silica gel G; Solvent, chloroform-acetic acid-water (4:1:1). Faint spots are outlined with dots.

(personal communication with Dr. G. M. Barton, Forest Products Laboratory, Department of Forestry and Rural Development of Canada, Vancouver, British Columbia). Phenolic compounds have not been mentioned in the literature for roots of Douglas-fir and red alder.

Ferulic acid was found to be inhibitory to P. weirii growth in vitro, while vanillic acid and caffeic acid were only weakly inhibitory (Table 9). It appears that ferulic acid in red alder roots is closely associated with the natural resistance to P. weirii infection. It is also possible that ferulic acid and vanillic acid possess synergistic activity against this fungus. Vanillic acid in the alder-conifer mixed stand soil could have been derived from two sources: vanillic acid in roots and litter of both Douglas-fir and red alder, or as an oxidation product of ferulic acid from red alder roots. The presence of greater amounts of vanillic acid in the interplanted stand soil could reasonably provide a chemical barrier to minimize the incidence of root-rot disease of conifers caused by P. weirii.

Petroleum Ether Leaf Extracts of Red Alder and Douglas-fir

Petroleum ether leaf extracts of red alder yielded three distinct compounds on thin-layer plates (Figure 15). Two of the compounds were identical to those of Douglas-fir with regard to colors under ultraviolet light (350 mμ) and daylight. The compound with Rf

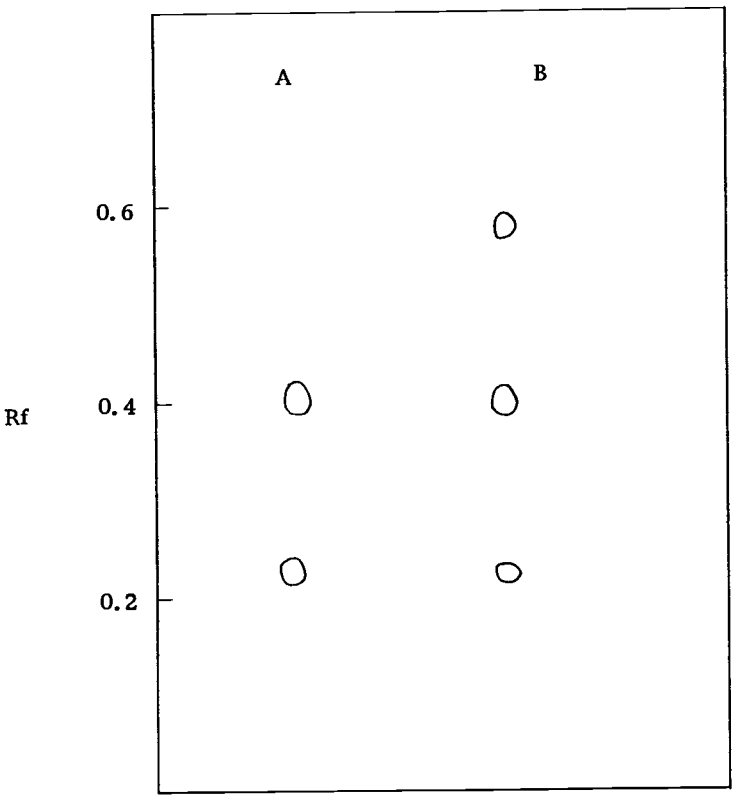


Figure 15. Chromatographic pattern of petroleum ether extracts of red alder and Douglas-fir leaves: A, Douglas-fir; B, red alder. Thin-layer, silica gel F_{254}; solvent, benzene-ethyl acetate (9:1).

value 0. 58 was dull green under ultraviolet light and bright yellow in daylight. The other two compounds with Rf values of 0. 40 and 0. 23 were dull red under ultraviolet light and light green in daylight. The particular compound obtained from red alder inhibited growth of P. weirii T-124 in vitro, particularly in the higher concentrations (Figure 16). Since the addition of ethanol to the medium varied with the concentration of compound added, and ethanol could influence fungal growth (Table 9), mean organic matter from the mycelium was compared with the control and compound at a given concentration but not between concentrations of a given compound. The absorbance spectrum of this compound in methanol gave three peaks: 475 mμ, 442 mμ, and 260 mμ (Figure 17). This experiment was designed to isolate coumarin compounds from red alder, as they have been reported to be widely distributed in various plant families and rarely, if ever, found in conifers (Soine 1964). The results indicate that the inhibitory compound in red alder leaves is not a coumarin compound.

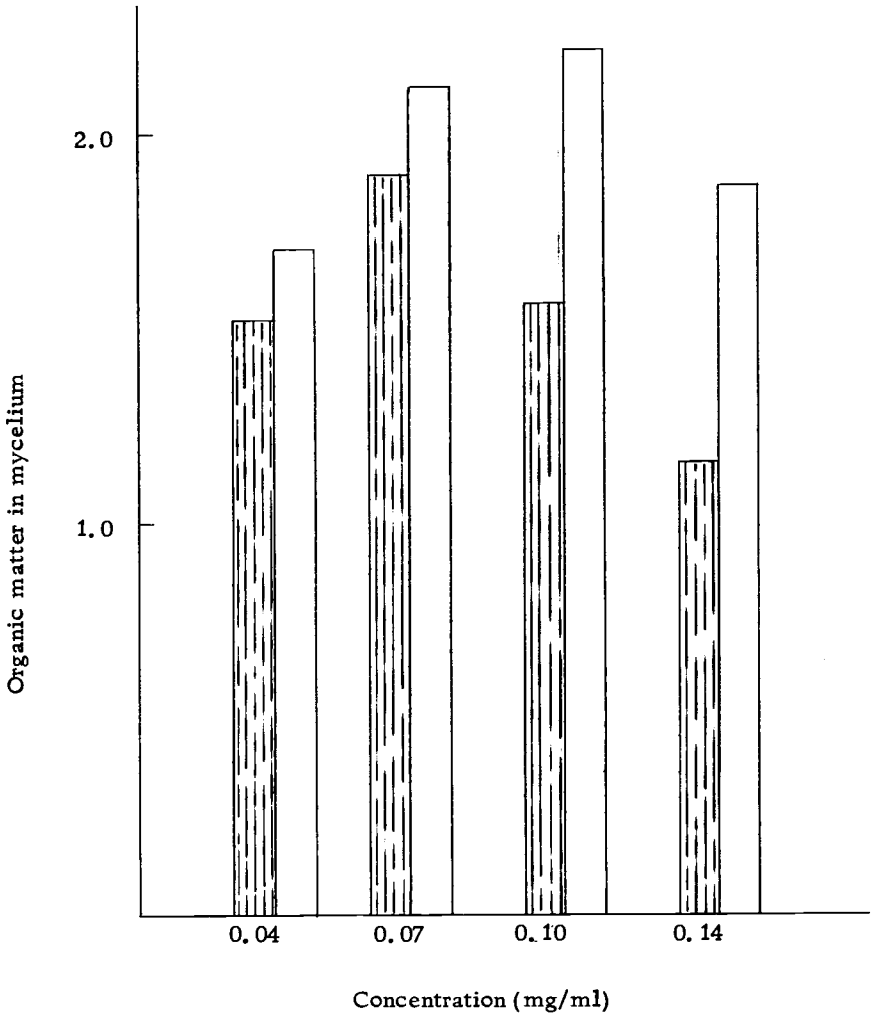


Figure 16. Effect of compound with Rf value 0.58 obtained from red alder leaves on growth of Poria weirii T-124 in vitro. Bar without shade is control. Organic matter in mg is mean of three replicates.

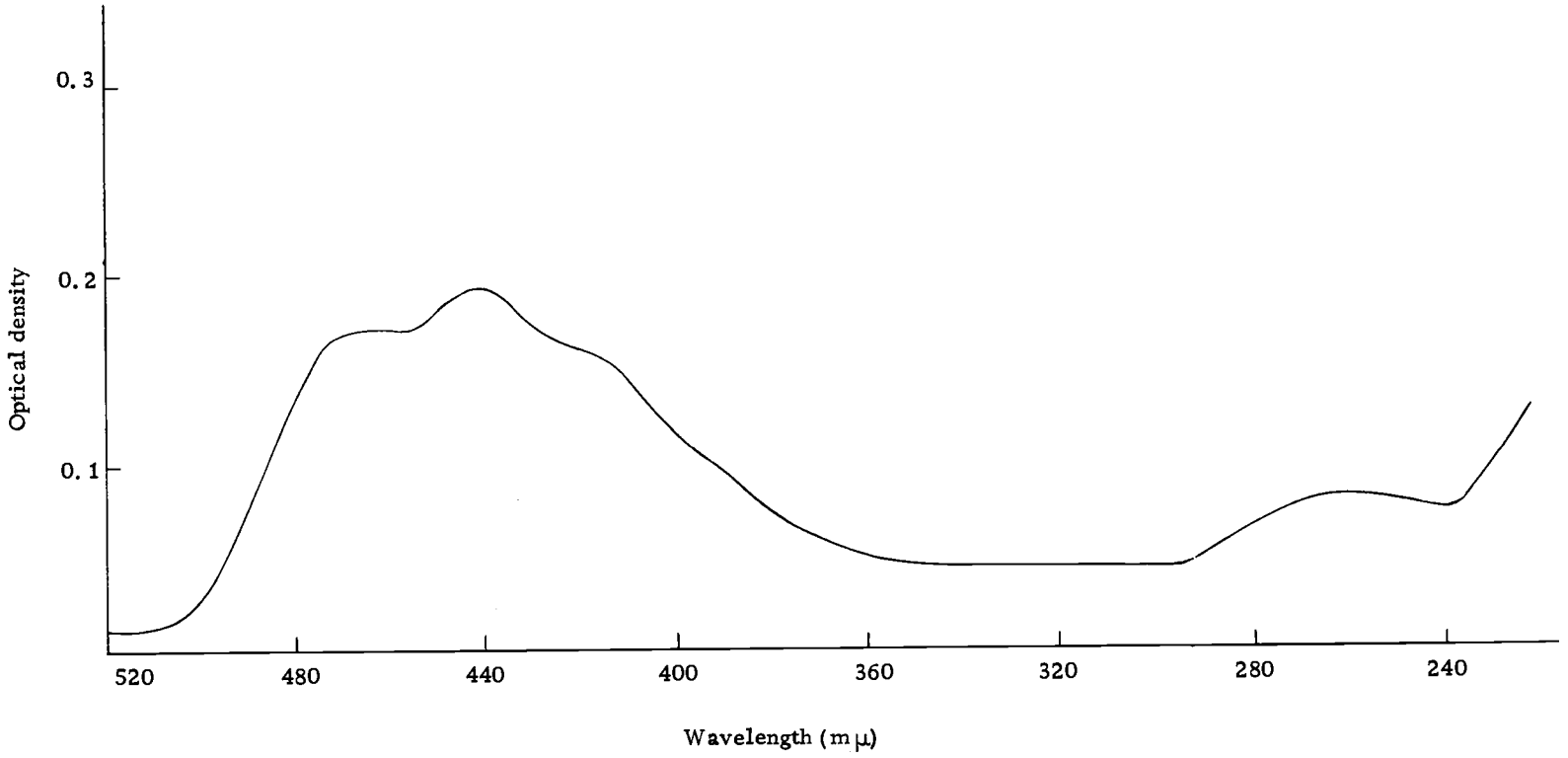


Figure 17. Absorption spectrum of compound with Rf value 0.58 obtained from red alder leaves.

SUMMARY AND CONCLUSION

Poria weirii causes heavy damage to coniferous timber stands in western North America. In Washington and Oregon alone, 32 million cubic feet are lost to this disease annually.

Red alder, a native, nitrogen-fixing hardwood, is highly resistant to P. weirii and in addition may discourage its development in stands containing red alder. Being resistant to infection, alder provides a mechanical barrier to P. weirii which spreads by growth along host roots from tree to tree. In addition, the studies reported here indicated possible mechanisms for the suppressing effects of alder on pathogenicity of P. weirii in mixed alder-conifer stands.

The high level of nitrate nitrogen in soils under red alder might easily affect disease development. Poria weirii was shown unable to utilize this form of nitrogen while many organisms antagonistic to this fungus could. The demonstrated lack of the enzyme, nitrate reductase, in P. weirii would seem to put the pathogen at definite competitive disadvantage with microorganisms possessing nitrate reductase in soils where the nitrate level is substantial. The percentage of antagonistic fungi and Streptomyces was shown to be considerably higher in soils under stands containing alder.

Temperature and pH ranges and optima for development of P. weirii as determined in this study were probably not sufficiently

different under the three stands examined to effect growth and development of the pathogen. Poria weirii grew quite well at pH values from 3.0 to 6.0 and at temperatures of 25° C or lower.

Poria weirii possesses extracellular oxidative enzymes, presumably phenoloxidases: o-diphenol oxidase, which catalyzes oxidation of catechol and DL-dopa; and p-diphenol oxidase, which catalyzes the oxidation of hydroquinone. These enzymes were unable to oxidize tyrosine, p-cresol, and p-phenylenediamine. The presence of phenoloxidases was assumed necessary for penetration of the pathogen into both conifer and alder roots. Red alder leaves and roots possess phenoloxidases which appear to be lacking in Douglas-fir leaves and roots. Peroxidase was present in both trees but at much higher levels in alder.

The resistance of red alder to P. weirii might be explained by the following hypothesis: On the penetration by the fungus, the phenolic compounds in alder tissues would be oxidized into fungitoxic compounds through catalytic action of phenoloxidases. These compounds deposited about the periphery of the penetrated area would further inhibit spread of the fungus and oxidized phenol would destroy or inactivate the fungal extracellular enzymes. The relatively high peroxidase activity may further contribute to resistance to the fungus.

Twenty-five phenolic compounds widely distributed in nature,

were tested individually in vitro at concentrations of 0.5 and 2.0 mM for effect on two isolates of P. weirii, T-124 and T-55. Growth of both isolates was strongly inhibited in a medium containing coumarin, 4-hydroxycoumarin, or 7-hydroxycoumarin at either concentration, but o-catechol, salicylic acid, benzoic acid, ferulic acid, o-coumaric acid, and phenylacetic acid were inhibitory only at the higher concentration. The remaining compounds either inhibited only one isolate, had no effect, or stimulated growth. At the 2.0 mM concentration, chlorogenic acid and gallic acid which were stimulatory to T-124 growth also stimulated the endogenous respiration of this isolate. Phenylacetic acid, salicylic acid, and coumarin at the same concentrations which were inhibitory to this isolate depressed the endogenous respiration. Linoleic acid in red alder was inhibitory to both P. weirii and Fomes annosus.

Total phenolics (tannins), expressed as tannic acid equivalent, in roots, litter, and soil of red alder, were two times as much as in roots, litter, and soil of Douglas-fir. In fresh leaves of alder total phenolics were much higher than in leaves of Douglas-fir. Tannins in soil under the mixed alder-conifer stand were higher than in soil of either pure stand. Tannic acid at higher concentrations inhibited growth of P. weirii on malt agar.

Ferulic and vanillic acid were identified in red alder roots, but only vanillic acid was found in Douglas-fir roots. Vanillic acid

was present in litter from both trees. Caffeic and vanillic acids were found in fresh leaves of red alder. Soil from under conifers, red alder, and mixed alder-conifer stands contained vanillic acid, but only in trace amounts under the conifer stand. It appears that ferulic acid in roots of red alder is associated with natural resistance to P. weirii. The presence of a greater amount of vanillic acid in the alder-conifer stand soil may provide a chemical barrier to minimize the incidence of root-rot disease of conifers caused by P. weirii.

An unknown compound inhibitory to P. weirii was found in petroleum ether extracts of fresh red alder leaves. It had absorbance peaks in methanol at 475 mμ, 442 mμ and 260 mμ. It was dull green under ultraviolet light and bright yellow in daylight, and had a Rf value of 0.58 on thin-layer silica get F_{254} with a benzene-ethyl acetate (9:1) solvent system. This particular compound was not found in leaves of Douglas-fir.

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