

AN ABSTRACT OF THE THESIS OF

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Title: RHIZOSPHERE MICROFLORAS ASSOCIATED WITH
MYCORRHIZAE OF DOUGLAS-FIR AND RED ALDER

Abstract approved [REDACTED]
Dr. Walter B. Bollen

The mycorrhizal rootlets of Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco.) and red alder (Alnus rubra Bong.) were extensively investigated. A jet-black mycorrhiza was found to be dominant on Douglas-fir rootlets. The fungal symbiont was identified as Cenococcum graniforme (Sow.) Ferd. and Winge. Two forms of mycorrhizae predominated on root systems of red alder. Detailed morphological studies, the first for this alder species, revealed distinct characteristic differences between the fungal symbionts. One common mycorrhizal form was clavate with a dark-brown roughened fungal mantle. The Hartig net was well developed. The other predominant form was pale brown and glabrous. The Hartig net was weakly developed and sporadic.

Rhizosphere microflora of three morphologically different mycorrhizae of a Douglas-fir were examined and compared with microflora surrounding adjacent suberized roots and with that in

nonrhizosphere soil. Populations of bacteria, molds, and Streptomyces were different for each microhabitat. Bacteria varied in total numbers and in distribution of morphological and physiological types. A low percentage of Streptomyces and fewer molds were found in mycorrhizal and adjacent suberized root rhizospheres than in the soil mass. Rhizosphere fungi were predominantly members of the genus Penicillium, which also were abundant, together with representatives of Aspergillus and Trichoderma, in nonrhizosphere soil. Rhizosphere differences between the morphologically distinct white, gray, and yellow mycorrhizae are attributed to influence of the different associated fungal symbionts.

Rhizosphere microfloras of Cenococcum graniforme mycorrhizae of Douglas-fir, of one type of ectotrophic mycorrhiza of red alder, and of nonmycorrhizal suberized roots of both tree species were investigated. Microbial populations and the most probable numbers of ammonifying and nitrate reducing microbes differed qualitatively and quantitatively among rhizosphere microhabitats. Nutritional classification of isolates from each rhizosphere microhabitat revealed that a large proportion was capable of synthesizing their growth requirements from glucose-salts media or from basal media supplemented with amino acids. In contrast, complex substances in yeast and soil extracts were required for growth by most of nonrhizosphere soil isolates. In manometric studies, homogenized

Douglas-fir nonmycorrhizal suberized root and red alder mycorrhizal root suspensions highly stimulated respiration of nonrhizosphere microbes, especially in the presence of glucose. Glucose oxidation, however, was suppressed in the presence of Douglas-fir mycorrhizal root suspension, probably by the antibiotic which the fungal symbiont, Cenococcum graniforme, is reported to produce. Glucose oxidation by nonrhizosphere microbes was similarly repressed in the presence of red alder nonmycorrhizal root suspension. An antibacterial substance, found in red alder root and nodule suspensions, inhibited growth of Bacillus subtilis and B. cereus on glucose-salts agar. These experimental results are discussed with reference to the influence of mycorrhizal and adjacent nonmycorrhizal suberized roots upon rhizosphere microfloras.

Rhizosphere Microfloras Associated
with Mycorrhizae of Douglas-fir
and Red Alder

by

John Lloyd Neal, Jr.

A THESIS

submitted to

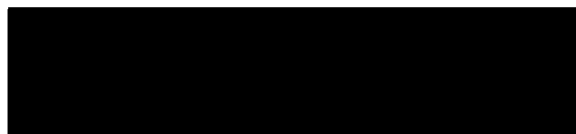
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APPROVED:



Professor of Microbiology
in charge of major



Chairman of the Department of Microbiology



Dean of Graduate School

Date thesis is presented September 5, 1967

Typed by Gwendolyn Hansen for John Lloyd Neal, Jr.

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RHIZOSPHERE MICROFLORAS ASSOCIATED WITH MYCORRHIZAE OF DOUGLAS-FIR AND RED ALDER

INTRODUCTION

Root hairs, as well as root surfaces, are covered with a mantle of microorganisms which can influence plant growth and welfare in many ways. The concentration of soil microorganisms upon plant root surfaces is intense, and at times may reach such proportions that microbial populations may exceed those of adjacent soil by 100 times. It is in this region, designated the rhizosphere, that soil microorganisms are able to exert the most direct influence upon plants. In the rhizosphere, beneficial products of microbial decomposition and synthesis, as well as toxic or injurious substances, may be absorbed at the plant root--soil interface. Additionally, competition for plant nutrients by the rhizosphere microbes influences general plant vigor and the amounts and kinds of nutrients that are absorbed by plant roots. Likewise, plants are able to exert profound stimulatory or inhibitory effects upon the rhizosphere microorganisms by metabolic excretions, thus favoring the development of specific microbial groups or species in the rhizosphere and, in some cases, forming a biological barrier to other soil microorganisms.

These concepts take on greater significance when viewed in relation to mycorrhizal roots and biological control of root rot

diseases in forest soils. Analysis of the rhizosphere microfloras associated with coniferous roots necessitates an investigation and consideration of what influence mycorrhizae have upon these microbes. Mycorrhizae, defined as a symbiotic fungal-root union, are so prevalent on tree roots in nature that nonmycorrhizal roots are rarely found.

With these concepts in mind, the present ecological study of rhizosphere microfloras associated with mycorrhizae was conducted as part of a broad investigation of forest soil microbes in relation to root rot.

The objectives of the study are as follows: (1) to determine if the rhizosphere microfloras of morphologically distinct mycorrhizae of a single Douglas-fir are qualitatively influenced by the fungal symbionts; (2) to ascertain if the qualitative influences are not also quantitative with particular reference to the antibiotic-producing mycorrhizal fungus, Cenococcum graniforme, commonly found on Douglas-fir rootlets; and (3) to determine if these influences in the rhizosphere are also prevalent with a dominant mycorrhiza of another common Oregon coastal tree, red alder (Alnus rubra Bong.). In order to obtain these objectives, it was necessary to extensively investigate gross morphology and rootlet anatomy of mycorrhizae associated with Douglas-fir and red alder. This area of research was found to be highly complex, demanding specialized techniques

and much time. Aside from the general microbiological aspects of the problem, it required learning to recognize the mycorrhizal root structures in the field and laboratory, learning the details of mycorrhizal rootlet anatomy, and obtaining a practical working knowledge of the general field.

As with many biological studies, more questions were often raised than were answered in this study. However, the results are reported with the intent of laying the basic groundwork for additional research in a heretofore little-investigated area of rhizosphere studies.

LITERATURE REVIEW

Sixty-three years have passed since Hiltner (1904) first introduced the term "rhizosphere" to designate the region of soil which is subject to the immediate influence of plant roots. He noted the rhizosphere soil supported greater microbial numbers and activity than soil more distant from plant roots. This observation has since been verified many times.

Since Hiltner's early work, a tremendous amount of data has been published about rhizosphere phenomena common to a variety of plants. The number of publications is conservatively estimated to exceed 500. It is beyond the scope of this thesis to include a detailed summary of each of these publications; however, a brief summation of the significant findings is in order.

It has been almost invariably found that in the rhizosphere, bacteria respond in activity and total numbers much more to the presence of plant root than do actinomycetes, fungi, algae, and protozoa. The root appears to apply a selective influence on the general soil microbial population via root exudates, metabolites, and sloughed-off cells. Most comparative studies show a definite selective stimulation by plant root of Gram-negative rods, physiologically active organisms, and amino acid requiring microorganisms.

Nutritional requirement studies have shown that most rhizosphere isolates grow on a basal medium of glucose, nitrate, and salts, or require amino acids, while most nonrhizosphere soil isolates require complex growth factors for minimal growth.

Taxonomic classification of bacteria isolated from the rhizosphere has proved quite difficult because many are atypical. Of those that have been classified, the majority belong to the genera Pseudomonas, Arthrobacter, Mycoplana, Micromonospora, Microbacterium, Corynebacterium, Bacillus, and "gram-negative" Norcardia.

Generally, a higher proportion of ammonifying bacteria are found in the rhizosphere than nonrhizosphere soil. Nitrifying microorganisms are not inhibited in the rhizosphere, but their activity is quite low. This is attributed to the rapid use of ammonium nitrogen by the plant and general rhizosphere microflora. Usually, great numbers of denitrifying bacteria are found in rhizosphere soil, but the extent of their activity is not clear. Much effort has been devoted to the establishment of selected bacterial species in the rhizosphere, particularly Azotobacter, but these attempts have had little success.

Evidence does exist that the rhizosphere harbors considerable numbers of phosphate-dissolving bacteria and that their activity increases the supply of available phosphate to the plant. However, the results of these studies are conflicting and await clarification.

Fungi are usually reported to be stimulated to a lesser degree by plant roots than are bacteria, but this is based upon total numbers of cells or propaguls, and not fungal cell volume or mass.

Many factors have been considered to have influence on the numbers or types of organisms that colonize a plant root. Among these are organic root exudates, decaying root materials, oxygen and carbon dioxide concentrations, changes in pH, water, mineral nutrients, plant species, and age. Of these, the first two are generally believed to exert the greatest effect.

Little is known about the influence that rhizosphere microbes may have upon general plant growth and vigor. There is evidence to indicate that rhizosphere microorganisms via their metabolites and activities influence root morphology, and root-to-shoot weight ratio; calcium, rubidium, phosphorus, sulfur, and nitrogen uptake; and certain specific physiological plant processes.

Additional information is given in excellent reviews by Katznelson, Lochhead and Timonin (1948); Clark (1949); Starkey (1958); and more recently by Rovira (1965a); and Timonin (1965).

In spite of the vast quantity of published material dealing with the rhizosphere effect of many plants, little information is available concerning the influence of mycorrhizae on the rhizosphere microflora and their activities.

Tribunskaya (1955) investigated the rhizosphere microflora of 5-month-old pine seedlings lacking or having poorly developed mycorrhizae in comparison with seedlings of similar age with well developed mycorrhizal structures. She found nine to ten times more fungi and greater numbers of proteolytic and fluorescent bacteria in the rhizosphere of mycorrhizal pine seedlings than in that of non-mycorrhizal seedlings. Her conclusions were that in addition to directly affecting the tree, the fungal symbiont influences the character of the rhizosphere microflora.

Mycorrhizal roots as compared to nonmycorrhizal roots of yellow birch were reported by Katznelson, Rouatt, and Peterson (1962) to have distinctive quantitative and qualitative differences. They observed numerically higher bacterial and actinomycete populations in the rhizosphere of mycorrhizal rootlets, but the numbers of fungi were lower than for nonmycorrhizal rootlets. Methylene-blue-reducing, ammonifying, and sugar fermenting bacteria were more strongly stimulated by mycorrhizal than by nonmycorrhizal roots.

The influence of mycorrhizal fungi upon the rhizosphere was further demonstrated by Neal, Bollen, and Zak (1964). The rhizosphere microflora of three morphologically distinct mycorrhiza, sometimes side by side on the same rootlet, and adjacent suberized roots of a single Douglas-fir were analyzed with reference to

nonrhizosphere soil microflora. Each mycorrhizal rhizosphere was found to contain significantly different populations of bacteria, fungi, and Streptomyces. Different morphological and physiological microbial groups predominated in each microhabitat as compared to each other and to nonrhizosphere soil. Differences in each rhizosphere were attributed to the type of fungal symbiont present.

EXPERIMENTAL METHODS AND MATERIALS

Mycorrhizal Studies

Mycorrhizal rootlets of red alder and Douglas-fir (Pseudo-tsuga menziesii (Mirb.) Franco) were collected in early autumn 1964, and in spring and autumn 1965, from a pure alder stand and a mixed conifer stand lacking alder, on the Cascade Head Experimental Forest near the town of Otis Junc., not far from the northern Oregon Coast. Samples were stored at 1°C and examined within 24 hours after collection.

The mycorrhizal root tips were severed from the main roots, cleaned ultrasonically, and fixed in chrome-acetic acid solution. Subsequently, the rootlets were paraffin-embedded, sectioned at 8 to 10 μ thickness, stained with safranin-fast green, and examined microscopically. Fresh whole specimens were saved for gross morphological examinations.

Nonmycorrhizal rootlets of red alder, desired as a base for evaluating effects of mycorrhizal infection on rootlet anatomy, could not be found in the stand. Accordingly, red alder seeds were surface sterilized, germinated, and grown aseptically, to obtain infection-free roots (Neal, Trappe, Lu, and Bollen, 1967b). Mycorrhizae-free rootlets were stained and sectioned as described in the preceding paragraphs.

Sections of nonmycorrhizal Douglas-fir rootlets were kindly furnished by Dr. J. M. Trappe, Forestry Sciences Laboratory, U. S. Department of Agriculture, Corvallis, Oregon for comparative study.

Rhizosphere Studies

Sampling Procedures

Ectotrophic mycorrhizae, suberized roots, and adjacent non-rhizosphere soil were collected in autumn 1962 and in spring 1963 from a large Douglas-fir tree about 40 years old in a predominately Douglas-fir and hemlock stand in the Siuslaw National Forest near the northern Oregon Coast.

Three different kinds of mycorrhizal structures, often occurring on the same rootlet, were selected on the basis of their morphology and color: white, yellow, and blue-gray (Figure 1). Clusters of mycorrhizae and adjacent suberized roots were severed from the root system and each placed into sterile capped tubes for transportation to the laboratory. Six samples of each mycorrhizal type, suberized root, and nonrhizosphere soil were analyzed independently. Sampling was limited to the surface eight inches of the soil stratum. Suberized roots of Douglas-fir were also included in the sampling in lieu of nonmycorrhizal root tips which could not be found.

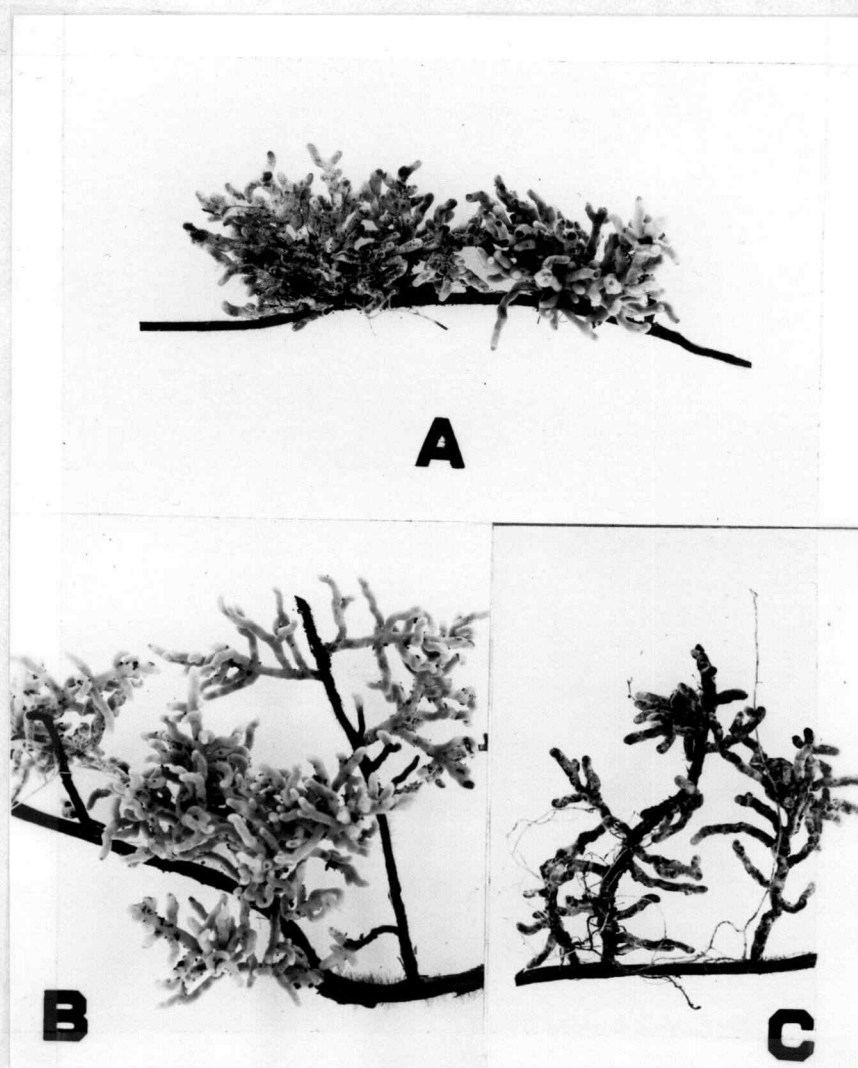


Figure 1. Two morphologically different mycorrhizae of Douglas-fir (X2.5). (A) Yellow and gray form side by side on the same rootlet. (B) Gray mycorrhiza; note smooth surfaces of rootlet. (C) Yellow mycorrhiza, showing coarse, reticulate surfaces and rhizomorphs of fungal symbionts.

Additional ectotrophic mycorrhizae, suberized roots, and adjacent nonrhizosphere soil were collected in the spring and fall, 1965, from the upper eight inches of soil in a pure red alder stand and, for Douglas-fir, from a mixed conifer stand lacking alder located on the Cascade Head Experimental Forest.

The roots were examined and the rhizosphere soil of the dominant Douglas-fir mycorrhiza, formed by Cenococcum graniforme, commonly found on Douglas-fir rootlets throughout the Pacific Northwest (Trappe, 1962) was analyzed in comparison to a predominant red alder mycorrhiza formed by an unidentified Basidiomycete (Neal, Trappe, Lu, and Bollen, 1967a). Three rhizosphere samples of each ectotrophic mycorrhizal type, adjacent suberized roots, and nonrhizosphere soil of Douglas-fir and red alder were analyzed independently.

Microbial Population Estimates

The mycorrhizal and adjacent suberized roots were freed of adhering soil by vigorous shaking, suspended in 10.0 ml of sterile water, and serially diluted. Appropriate 0.1 ml aliquots were spread over the surface of hardened agar plates. A glass rod bent at right angles was used to spread the 0.1 ml soil-in-water suspension (Neal, Lu, Bollen, and Trappe, 1967) on soil extract agar (Allen, 1957) for bacteria, on Martin's (1950) rose bengal agar for

molds, and on sodium albuminate agar (Waksman, 1922) for Streptomyces. The plates were incubated at 28°C seven days for bacteria and mold and twelve days for Streptomyces.

The amount of soil for each rhizosphere sample was estimated by obtaining the oven-dry soil weight in a 2.0 ml aliquot of the soil-water suspension; the accuracy of this method was checked using known weights of oven-dry soil in 100 ml suspensions.

Microbial population estimates of 1:5 nonrhizosphere soil-water suspensions were obtained in a like manner.

Approximately 200 bacterial colonies from rhizosphere and nonrhizosphere agar plates were transferred to nutrient broth enriched with 200 ml of soil extract per liter by sterile wooden tooth-picks for further study. The colonies were systematically selected so that all on a whole or a definite section of a plate were included.

Morphological Studies

Each of the isolated cultures was Gram stained by use of Kopeloff and Beerman's procedure (Society of American Bacteriologists, 1957) and classified according to staining reaction and morphological characteristics. Aerobic spore formers were determined in nutrient broth enriched with soil extract by plating a 1:5 suspension after pasteurization at 85°C for 10 minutes (Clark and Smith, 1950).

Physiological Studies

Ability of isolated microorganisms to produce ammonia, and to reduce nitrate to nitrite, was determined by inoculating 1% peptone water and 1% KNO_3 broth. After 7 days incubation at 28°C , ammonia was detected by Nessler's reagent; the presence of nitrites determined by Griess' reagent (Society of American Bacteriologists, 1957).

Quantitative estimates of ammonifying and of nitrate reducing bacteria were made by the Most Probable Number (MPN) technique using 10-fold dilution series with five replicate aliquot samples from each dilution (Alexander, 1965).

Nutritional Classification

Nutritional classification of microorganisms isolated from each microhabitat and nonrhizosphere soil was carried out on differential media according to the method of Lochhead (1940) as modified by Stevenson and Rouatt (1953), using the rapid replica plating technique described by Neal, Lu, Bollen, and Trappe (1966). The replicator was devised to reduce the tedium, multitude of different tubed media, and number of man-hours associated with a nutritional classification study.

The replicator consists of three main parts. The press is a bottle capper, modified to fit into a wooden frame (Figure 2A). The

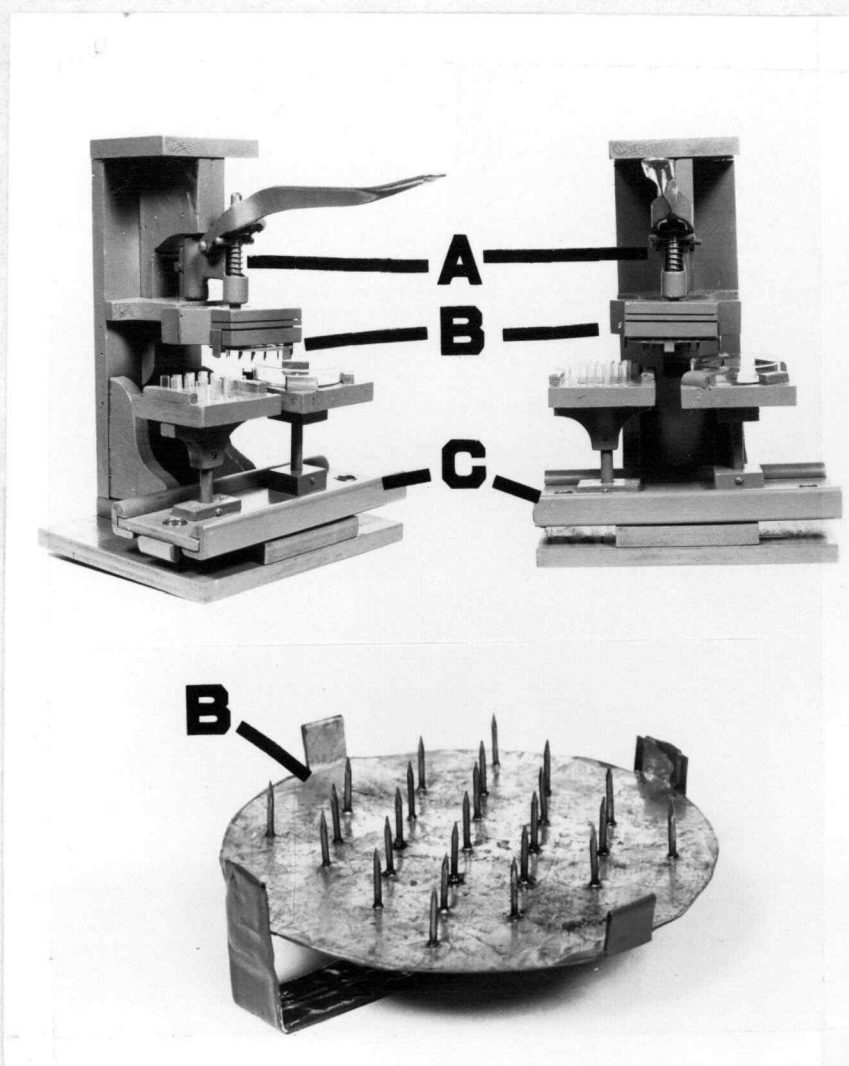


Figure 2. Replicating inoculator. (A) Press, with block to hold inoculating needle. (B) Inoculating needles soldered in metal disk. (C) Sliding platform supporting rack of culture tubes and petri dish to be inoculated.

replicating part (Figure 2B) is cut to the size of a petri dish from 16-gauge galvanized sheet metal. Brass nails (1.3 cm long) were inserted through and soldered in small holes drilled through the metal, 1.3 cm apart. The third part (Figure 2C) consists of a sliding platform supporting two wood stands. One functions as a culture tube rack, the tubes being spaced 1.3 cm apart to match position of the brass nails. The other stand holds the petri dish into which the nails carrying inocula are to be imprinted. To prevent contamination, a sterile petri-dish lid covers the array of culture tubes when not in replicating position. In operation, 1 ml culture tubes containing suspensions of isolates obtained by standard methods are slid to the right under the metal replicator which is then depressed, each brass nail serving as an inoculation needle. The culture tubes are then covered with the protective petri lid, and the stand holding the dish to be inoculated is then slid to the left under the replicator. Depression of the replicator imprints the agar medium with an inoculum of each culture. Replication of 25 cultures occurs simultaneously; the process may be repeated as often as desired.

The metal replicator may be autoclaved or flamed with alcohol when cultures are changed. Flat-bottomed tubes (8 by 30 mm) were best suited for the replicator. Contamination, readily detected on

inoculated plates by position out of the nail pattern, is minimal, especially when plates are replicated in a culture hood.

The microorganisms were placed in seven groups on the basis of growth in the following nutritional medium.

- I. Basal Medium (B) - glucose-salts agar. Bacteria capable of growth in the medium are able to synthesize their requirements for growth from glucose-nitrate-salts medium and therefore have simple requirements.
- II. Medium A - Basal medium plus Bacto Casamino Acids. Organisms growing in this medium, but showing no growth on medium B, require one or more amino acids for development.
- III. Medium G - Basal medium plus growth factors. Bacteria growing in this medium, but showing no growth on medium B or A, require vitamins for growth.
- IV. Medium AG - Basal medium plus amino acids and growth factors. Bacteria growing in the medium, but showing no growth in medium B, A, or G, require in addition to one or more growth factors, one or more amino acids for development.
- V. Medium Y - Basal medium plus yeast extract. Bacteria growing in this medium, but showing no growth in medium

B, A, G, or AG, require unidentified substances in yeast for growth.

VI. Medium S - Basal medium plus soil extract. Bacteria growing in this medium, but showing no growth in medium B, A, G, AG, or Y, require unidentified substances in soil extract for development.

VII. Medium YS - Basal medium plus yeast and soil extracts. These bacteria are capable of growth only in medium YS. In all other medium, growth is absent. The results are reported as the percentage of total isolates capable of growth in the different medium.

Warburg Studies

In metabolic studies, respiration was measured by the direct method (Umbreit, Burris, and Stauffer, 1957). Mycorrhizal and adjacent suberized roots from Douglas-fir and red alder, respectively, were severed from the main roots, cleaned ultrasonically, surface sterilized in 2.0% sodium hypochlorite solution for five minutes, and serially washed with sterile distilled water. The roots were homogenized by a Carver hydraulic press at 10,000 psi. The root slurry was collected aseptically and adjusted with sterile 0.1 M phosphate buffer pH 6.5, to a final volume containing 20.0 mg of root material, oven-dry basis, per ml of root suspension. Ten grams,

oven-dry basis, of nonrhizosphere soil sieved through a 40-mesh screen were added to 100 ml of sterile 0.1 M phosphate buffer, pH 7.0. One ml of the resulting soil-buffer suspension, vigorously shaken, was added to each Warburg flask as inoculum. Each flask contained 0.5 ml of root suspension (10 mg of root material, oven-dry basis), 0.5 ml of 0.1 M phosphate buffer, pH 7.0, 0.2 ml of 20% KOH in the center well fitted with fluted filter paper to absorb carbon dioxide, and 0.5 ml of 0.1 M glucose in the flask sidearm. Appropriate controls of KOH with soil inoculum only, root material only, and root material plus glucose were included. A sufficient volume of 0.1 M phosphate buffer, pH 7.0, was added to each flask when needed to bring the total liquid volume to 3.0 ml. Duplicate flasks of each treatment were attached to calibrated manometers and placed in a constant temperature water bath held at 29.5°C. The flasks were allowed to equilibrate 1 hr before the manometers were closed. Glucose solution previously pipetted in the flask side arm was tipped in immediately after equilibration.

Biological Assay

Assays for biologically antagonistic substances in red alder roots and nodules were determined by the paper-disk plate method (Loo et al., 1945) using stock cultures of Bacillus cereus and B. subtilis. Root and nodule slurries were prepared as previously

described from material collected during the summer and winter months and concentrated approximately 50-fold by lyophilization. Paper disks were impregnated with the slurry suspensions and placed on peptone-beef extract (beef extract, 3 gm; peptone, 5 gm; distilled water, 1000 ml) and glucose-salts agar (glucose, 1.0 gm; K_2HPO_4 , 1.0 gm; KNO_3 , 0.5 gm; $MgSO_4$, 0.2 gm; $CaCl_2$, 0.1 gm; NaCl, 0.1 gm; $FeCl_3$, 0.01 gm; distilled water, 1000 ml) seeded with the test organisms and incubated for 48 hr at 30°C. Controls were paper disks impregnated with sterile distilled water.

Statistical Analyses

Statistical analyses included least significant at 5% level of significance (LSD 0.05) for comparing mean numbers of organisms. Comparisons were made separately for total bacteria, molds, and Streptomyces. In addition, a chi-square test for independence was used to determine if the proportions of total bacteria, molds, and Streptomyces were similar or dissimilar.

RESULTS AND DISCUSSION

Mycorrhizal Studies

To investigate the rhizosphere of Douglas-fir and red alder mycorrhizal rootlets, it was necessary to study in detail the pre-dominant fungal symbionts that were prevalent on the roots of each investigated tree species located in the study area.

Douglas-fir Mycorrhizae

Examination of Douglas-fir root collections from a mixed conifer stand lacking alder revealed jet-black mycorrhizae on about 95% of the root tips. Gross morphological characteristics of the mycorrhizae were compared to those described by Trappe (1962), and the mycorrhizal fungus was tentatively identified as Cenococcum graniforme. Sclerotia immeshed in the hyphal strands radiating from the mycorrhizae were surface sterilized using a Slankis apparatus (1958) as modified by Trappe (1962). Once surface sterilized, the sclerotia were transferred to tubed growth media (Melin and Mikola, 1948) and incubated at room temperature. Hyphae growing from the sclerotia after a suitable incubation period were identified as C. graniforme by Dr. J. M. Trappe, Forestry Sciences Laboratory, Corvallis, Oregon. Hyphae growing from sclerotia on

synthetic media were morphologically identical to those forming the jet black mycorrhiza on the root tips of Douglas-fir.

According to Trappe (1962), ectotrophic mycorrhizae formed by C. graniforme are found on a variety of conifers and hardwoods. The mycorrhizae formed by this fungal symbiont are reported to occur throughout most of Europe, continental United States, and southwestern Canada, as far north as the Arctic Circle in Alaska, and as far south as Mexico (Trappe and Quinard, 1966) and Puerto Rico.

C. graniforme as a mycorrhiza is found in a variety of habitats, some quite severe. For example, the fungus will thrive in extremely cold areas, marshes and ponded soils, sand dunes, desert soils, and subtropical areas.

A mycorrhiza formed by C. graniforme on Douglas-fir rootlets has distinct, easily identifiable morphological characteristics (Figure 3A). The mycorrhizal rootlet is jet black, usually not branched, or sometimes irregularly branched, and has numerous stiff projecting nonseptate black hyphae which radiate in all directions from the rootlet. The fungal mantle usually covers all or most of the short root. Mycorrhizal hyphae may extend several centimeters or meters from the rootlet, depending upon environmental conditions. In the area where the samples were collected, the soil

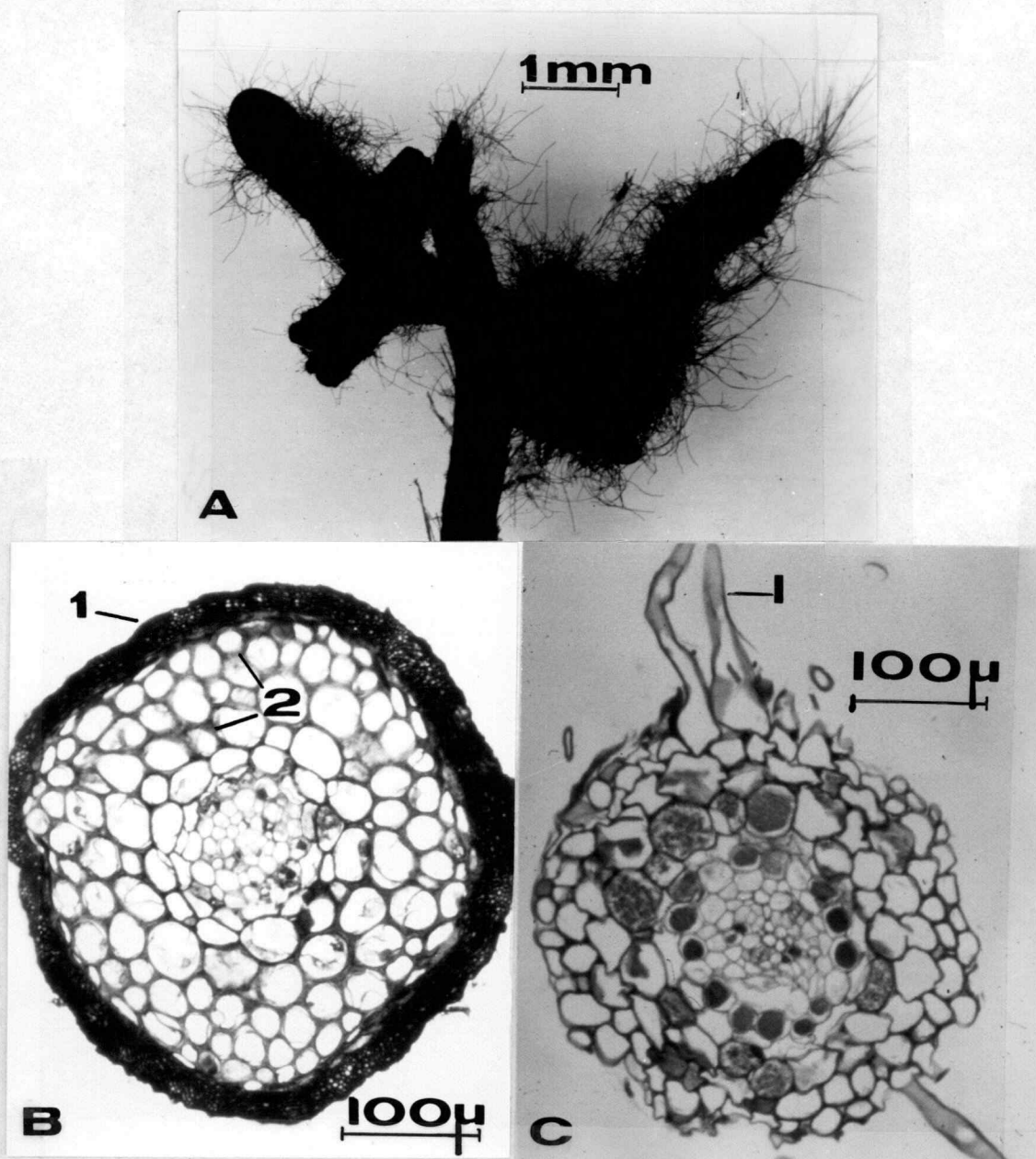


Figure 3. (A) Douglas-fir mycorrhizal rootlets formed by Cenococcum graniforme showing jet-black hyphae radiating from rootlet. (B) Cross-section of Douglas-fir rootlet infected with Cenococcum graniforme. (B-1) Fungal mantle (B-2) Hartig net. (C) Uninfected young Douglas-fir rootlet. (C-1) Root hair.

was quite dark and when closely examined, was permeated with numerous black hyphal strands.

A stained cross-section of a Douglas-fir mycorrhizal rootlet formed by C. graniforme is shown in Figure 3B. The fungal mantle is composed of thick walled hyphal cells which appear to radiate in all directions (Figure 3B-1). The mantle thickness ranged from 20 to 40 μ . The mantle tissue, because of its characteristic pattern, is called prosenchyma (Trappe, 1962).

The Hartig net (Figure 3B-2) hyphae penetrated intercellularly; no intracellular infection was observed. Hartig net hyphae averaged 2.5 μ in diameter. Cortical cells ranged in diameter from 30 to 50 μ . Xylem was diarch.

For comparative purposes, a cross-section of a young, uninfected Douglas-fir rootlet was examined (Figure 3C). Root hairs, absent on rootlets infected with the mycorrhizal fungus, projected from the outer cortical cells and were 5 to 10 μ broad (Figure 3C-1). Epidermal cells ranged in diameter from 5 to 7 μ by 6 to 10 μ in cross-section. Immature cortical cells underlying the epidermis ranged in diameter from 20 to 35 μ . The xylem was diarch.

The contrast between cross-sections of mycorrhizal and non-mycorrhizal rootlets of Douglas-fir is evident, the latter lacking a fungal mantle and Hartig net, but having root hairs and irregular shaped cortical cells.

Red Alder Mycorrhizae

Red alder is unique among the trees of the Pacific Coast because its roots participate in either one of two major kinds of symbiosis: root nodules formed with nitrogen-fixing endophytes, presumably Streptomyces, and mycorrhizae formed with certain fungi. This particular study of the rhizosphere phenomena associated with mycorrhizal roots of red alder led to the extensive investigation of its mycorrhizae.

The study represents the first detailed morphological study of mycorrhizae of red alder. Two forms of alder mycorrhizae predominated in the root collections from a pure alder stand located on the Cascade Head Experimental Forest. A third mycorrhizae formed occurred on the rootlets only infrequently, so it is not included in the study. No mycorrhizae were found to be formed by C. graniforme, the only type heretofore reported for red alder (Trappe, 1964).

One common form of mycorrhizae, comprising about 40% of those collected, was generally clavate with a dark-brown, distinctively roughened fungal mantle (Figure 4, left) which was commonly ruptured by apical growth of the enclosed rootlet (Figure 4, right). This mantle often was sloughed off when roots were removed from the soil.

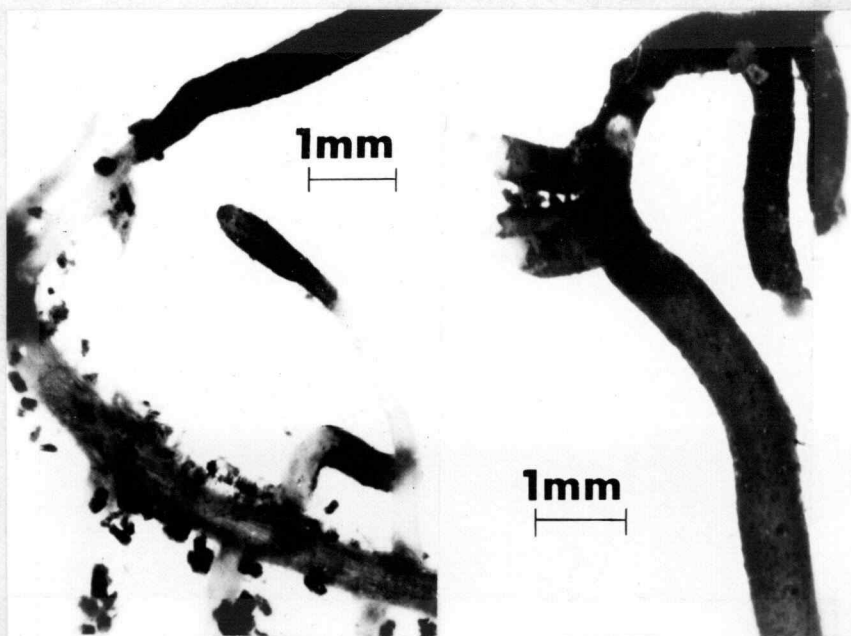


Figure 4. Red alder mycorrhizal rootlets formed by unidentified basidiomycete. (Left) Clavate red alder mycorrhiza with dark brown fungal mantle. (Right) Fungal sheaths of dark brown ruptured by apical growth of enclosed rootlet.

Two layers of tissue comprised the mantle (Figure 5, left), which totaled 40 to 60 μ in thickness. The outermost layer, 30 to 45 μ thick, was formed of irregular, more or less isodiametric, thick-walled cells 8 to 12 μ broad (Figure 5, left A). Irregular collapse of the peripheral cells of this layer accounts for the mycorrhiza's surface roughness. The inner mantle layer, 10 to 15 μ thick, was composed of thin-walled hyphae 2 to 5 μ in diameter, aligned predominantly along the root axis (Figure 5, left B). The Hartig net penetrated only the epidermis (Figure 5, left C), separating epidermal cells by one layer of hyphae 1.5 to 2.5 μ thick.

The outermost root cells ranged from 15 to 27 μ x 10 to 20 μ in cross-sectional diameter; cortical cells, 30 to 85 μ x 30 to 40 μ ; and endodermal cells, 10 to 20 μ . The xylem was triarch.

Septate hyphae, averaging about 5 μ in diameter and having walls about 1 μ thick, sometimes originated from turgid cells at the mantle surface (Figure 5, right). Clamp connections were present at some septa of these hyphae. Therefore, the fungus was a Basidiomycete.

The other common form of mycorrhiza (Figure 6, left), comprising about 50% of those collected, was pale brown and glabrous. No mycorrhizal mantle was obviously present under low-power magnification, but side-by-side comparison with uninfected roots revealed distinct differences, especially in color.

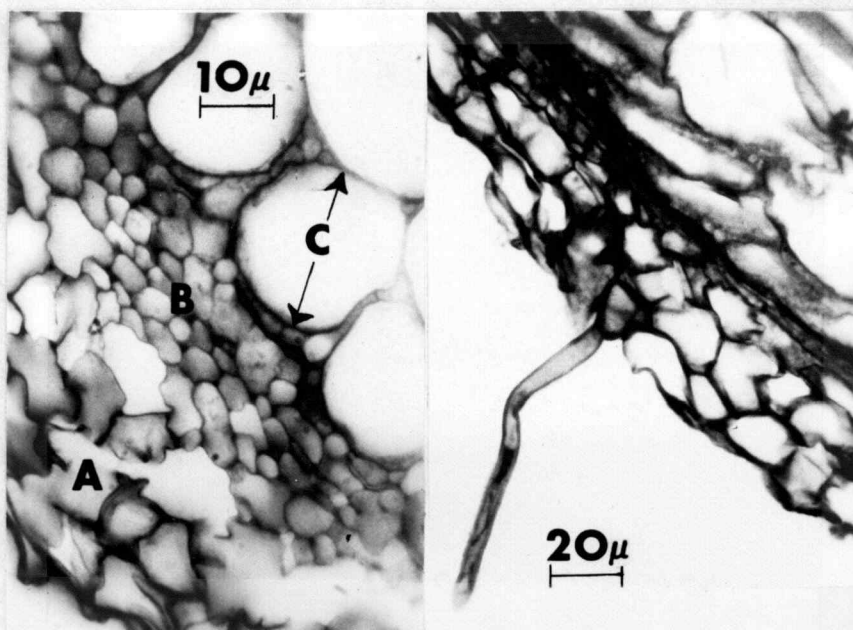


Figure 5. (Left) Cross-section of two-layered fungal mantle and outer root cortex of dark brown, clavate, red alder mycorrhiza. (A) Outer mantle layer. (B) Inner, smaller, densely packed inner mantle layer. (C) Hartig net formed by intercellular penetration of mantle hyphae. (Right) Longitudinal section showing hypha originating from outer mantle cell of dark brown mycorrhiza.

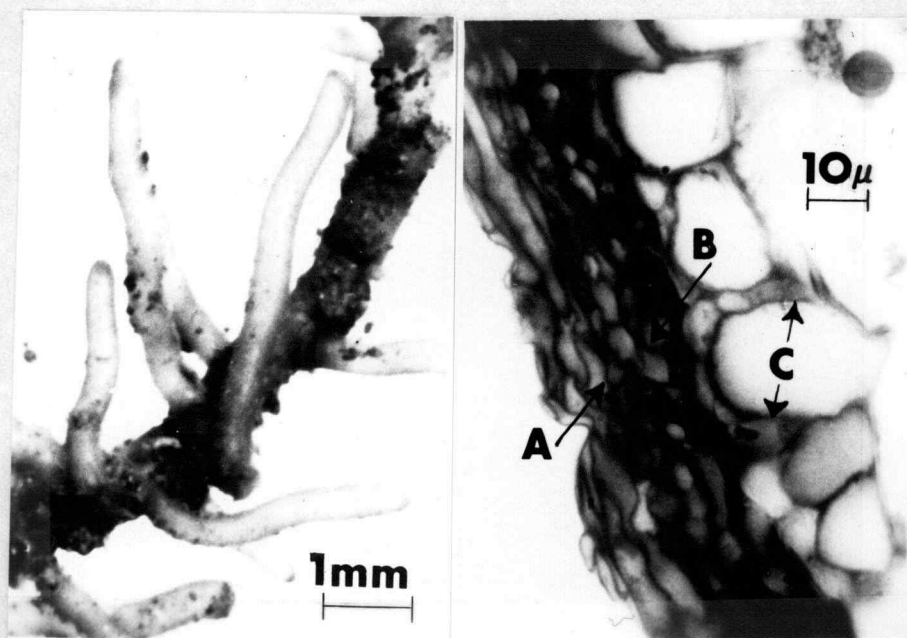


Figure 6. (Left) Red alder mycorrhiza with pale brown, glabrous mantle. (Right) Cross-section of mantle and outer root cortex of pale brown, glabrous mycorrhiza. (A) Thin single-layered mantle. (B) Opaque amorphous layer. (C) Vestige of Hartig net.

The mantle, 12 to 25 μ thick, constituted a single-layered prosenchyma tightly adhering to the rootlet surface and aligned predominantly with the root axis (Figure 6, right A). Mantle hyphae were 2 to 2.5 μ in diameter.

A single-layered Hartig net (Figure 6, right C), 2 to 3 μ broad, sporadically penetrated the rootlet epidermis. Consequently, this mycorrhiza is considered weakly ectotrophic, even though a true Hartig net was lacking from much of the fungus-root interface. A layer of opaque, amorphous, red-stained material, perhaps tannin, was often present between the epidermis and the mycorrhizal mantle (Figure 3, right B).

The outermost root cells varied from 10 to 30 μ in diameter; cortical cells, 30 to 65 μ ; and endodermal cells 9 to 18 μ . The xylem was triarch.

For lack of distinctive, morphological characteristics, the fungus participating in this form of mycorrhiza could not be classified.

The nonmycorrhizal rootlets had an epidermis of cells 5 to 10 μ x 8 to 16 μ in cross-sectional dimensions (Figure 7A). Root hairs 6 to 9 μ broad originated sporadically from this outer layer of cells (Figure 7B). Cortical cells underlying the epidermis ranged from 15 to 17 μ x 15 to 35 μ in diameter, and endodermal cells from 3 to 7 μ x 7 to 12 μ . The xylem was monarch.

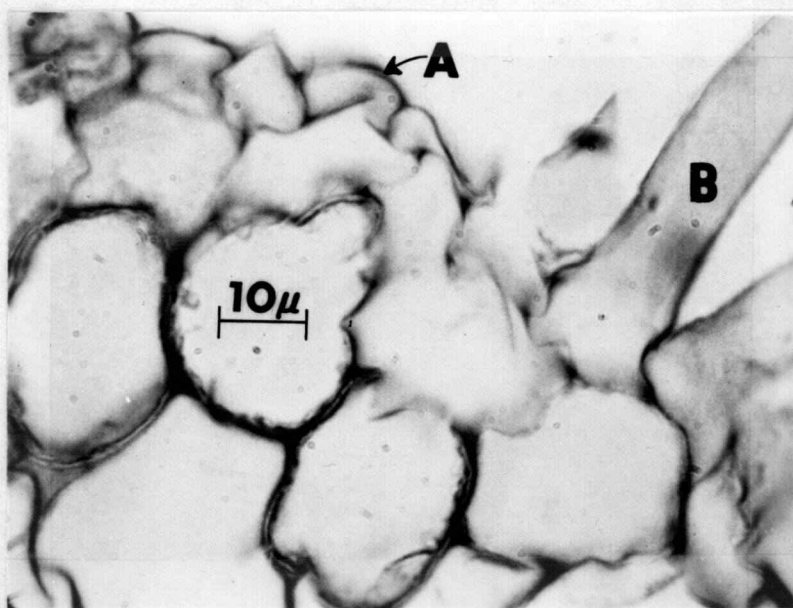


Figure 7. Cross-section of red alder mycorrhizae-free root tip. (A) Epidermal cells. (B) Root hair.

The lack of root hairs on the mycorrhizae is the only anatomical change that can be attributed to mycorrhizal infection; this conclusion is based on comparison between mycorrhizae collected from mature roots and nonmycorrhizal rootlets from seedlings grown aseptically. The outermost tier of root cells on the mycorrhizae, though smaller than the cortical cells, did not appear to be a typical epidermis in comparison with that of nonmycorrhizal roots. It cannot be said, however, whether this difference is due to mycorrhizal infection or whether it merely reflects the difference in age and environment of the mycorrhizal versus nonmycorrhizal roots. Masui (1926) illustrated a similar phenomenon in his paper on mycorrhizae of Japanese alders, as did Klecka and Vukolov (1936) for Alnus incana (L.) Moench.

No counterpart of the rough, brown mycorrhiza of red alder has been reported for other Alnus species, but the glabrous form resembles a mycorrhiza of Alnus incana in Europe (Klecka and Vukolov, 1935). All have a Hartig net that penetrates only the outer tier of root cells as did several other forms of ectotrophic mycorrhizae described for Japanese Alnus species (Masui, 1926). This shallow penetration by the Hartig net appears to be characteristic of Alnus species, in contrast to the deeper penetration frequent in ectotrophic mycorrhizae of many other tree species.

Sporocarps of fungi fruiting in the stand were collected over the course of 18 months (Neal, Trappe, Lu, and Bollen, 1967b). Of the 30 terrestrial species found, only 5 were likely to form mycorrhizae: Alpova cinnamoneus Dodge, Hymenogaster alnicola A. H. Smith, Lactarius obscuratus (Lasch) Fr., and 2 Inocybe species. It cannot be said with certainty if any of these is the fungal symbiont of either of the mycorrhizae described. Lactarius obscuratus, however, fruits abundantly in the stand and has basal hyphae similar to those forming the mantle of the glabrous mycorrhiza.

Because of their distinctive and easily recognized morphological characteristics, the rhizosphere soils around mycorrhizae formed by C. graniforme on Douglas-fir and mycorrhizae formed by an unidentified Basidiomycete on red alder were chosen for study.

Rhizosphere Studies

Microfloras of Morphologically Different Douglas-fir Mycorrhizae

The rhizosphere microbial population estimates of three morphologically distinct mycorrhizae (Figure 1) are shown in Table 1. Statistical analyses of the plate counts of bacteria and Streptomyces showed, except for Streptomyces in the white mycorrhiza rhizosphere as compared to nonrhizosphere soil, that populations surrounding each mycorrhizal formation and suberized root

Table 1. Microbial populations of rhizosphere and nonrhizosphere soil

Rhizosphere type	Bacteria			Molds
	Total millions ^a	Streptomyces millions ^a	Percent ^b	thousands ^a
Yellow mycorrhiza	325.0	10.0	3.0	64.0
White mycorrhiza	83.8	1.2	1.5	162.0
Gray mycorrhiza	206.0	5.3	2.6	200.0
Suberized root	183.0	24.2	13.2	183.0
Non-rhizosphere soil	6.3	1.3	20.4	238.0
L.S.D. 0.05	10.841	1.927		10.742

^aPer gram of soil, oven-dry basis. Each value represents the mean of six replicates.

^bPercentage of total bacteria, including Streptomyces.

were significantly greater than those of nonrhizosphere soil. These microbial increases in the rhizosphere agree with the findings of many investigations (Rovira, 1965a; Timonin, 1965; Clark, 1949) all of which report that total numbers of bacteria are greater in the vicinity of plant roots.

Significantly large differences were found between rhizosphere bacterial populations of the morphologically different mycorrhizal formations as compared to each other and the adjacent suberized roots.

The rhizosphere soil of yellow and gray mycorrhizae supported higher populations of bacteria than the suberized root rhizosphere. In all the microhabitats, with the exception of rhizosphere of white mycorrhizae, the Streptomyces population was significantly higher than in nonrhizosphere soil. The difference between the rhizosphere Streptomyces population of gray mycorrhizal rootlets and that of non-rhizosphere soil was not significant. The percentage of Streptomyces in the rhizosphere, based on the total bacterial population estimate, was considerably lower (Table 1). For example, the Streptomyces number 1.2 million per gram of soil (oven-dry basis) in the rhizosphere soil surrounding white mycorrhizal rootlets but the percentage of Streptomyces was only 3.1.

Although the Streptomyces numbered 1.3 million per gram in nonrhizosphere soil, their percentage of the total bacteria population was 20.4.

Mold counts were lower in mycorrhizal rhizosphere than in nonrhizosphere soil (Table 1). In all cases, the mold population was significantly different when total numbers in each microhabitat were compared to each other and to nonrhizosphere soil. The predominating molds in each rhizosphere were members of the genus Penicillium. This agrees with the findings reported by Katznelson, Rouatt, and Peterson (1962) for mycorrhizae of yellow birch. Representatives of this genus were also abundant in the nonrhizosphere soil, but numerous representatives of Aspergillus, Trichoderma, and Mucor were also present.

Colonies of yeasts were noted on the plates used to enumerate molds from the rhizosphere soil of yellow and gray mycorrhizae, none were evident in soils of the other microhabitats examined. In this connection, it is of interest to note that certain mycorrhizal Basidiomycetes have been obtained in culture by placing spores on agar plates on which colonies of yeasts, such as Torulopsis sanguinea, were present (Fries, 1942; Fries, 1943).

Frequency of isolation of bacteria from each rhizosphere and nonrhizosphere soil according to morphological types are shown in Table 2. Different predominating morphological groups were found

Table 2. Frequency (in %) of morphological types of bacteria in rhizosphere and nonrhizosphere soil^a

Rhizosphere type	Cocci	Ovoid rods	Rod-shaped bacteria ^b		Pleomorphic rods
			Non-spore forming	Spore forming	
Yellow mycorrhiza	11.5	42.3	32.3	14.0	9.7
White mycorrhiza	4.0	8.0	41.4	46.6	16.0
Gray mycorrhiza	1.6	72.2	18.2	18.0	0.0
Suberized root	10.0	43.3	22.1	24.6	11.7
Non-rhizosphere soil	0.0	36.1	43.3	20.6	5.6

^aEach value is based upon total number of isolants for each rhizosphere type.

^bIncluding pleomorphic rods.

to be associated with each microhabitat. While cocci were found in each rhizosphere, they were not present among isolates from non-rhizosphere soil. Pleomorphic rods were absent in the rhizosphere of gray mycorrhiza, but were abundant in all other microhabitats and nonrhizosphere soil. Spore formers, pleomorphic rods, and distinctly rod-shaped bacteria were most common in the rhizosphere of white mycorrhiza, while in the gray mycorrhizal rhizosphere ovoid rods were dominant with but few spore-formers and no pleomorphic rods. In general, different morphological types were found to predominate in each rhizosphere and nonrhizosphere soil.

The predominance of different morphological groups in the rhizosphere soil of each mycorrhiza may be attributed to type of fungal symbiont present.

Approximately 45% of the rhizosphere isolates were found to be Gram-positive, with the exception of the white mycorrhizal rhizosphere bacteria, among which gram-negative rods predominated.

Gram-negative bacteria also prevailed in the nonrhizosphere soil; the percentage being 63.9.

Comparison of the bacterial isolates according to ammonifying and nitrate reducing powers are shown in Table 3. Bacterial isolates producing ammonia from peptone and those reducing nitrates to nitrites in nitrate broth were more numerous in nonrhizosphere soil than in soil from each microhabitat. A higher percentage of

Table 3. Frequency (in %) of physiological types of bacteria in rhizosphere and nonrhizosphere soil^a

Rhizosphere type	Gram positive	Ammonifiers ^b	Nitrate reducers ^c
Yellow mycorrhiza	43.6	25.6	2.0
White mycorrhiza	20.0	30.0	40.6
Gray mycorrhiza	42.6	85.2	76.2
Suberized root	46.7	63.3	86.4
Non-rhizosphere soil	36.1	90.3	96.8

^aEach value is based upon total number of isolants for each rhizosphere type.

^bCapable of producing NH_4^+ in 1% peptone water.

^cCapable of reducing NO_3^- to NO_2^- in nitrate broth.

organisms capable of reducing nitrate to nitrite occurred in rhizosphere soil associated with the suberized root than in each mycorrhizal habitat. The rhizosphere of yellow mycorrhizae had the lowest percentage of ammonifiers and nitrate reducers, the latter rarely occurring in this microhabitat. Bacteria of these two physiological groups were proportionately much more numerous in the rhizosphere of white mycorrhizae and still more abundant in the rhizosphere of gray mycorrhizae. Gas production from nitrate was absent in all cases.

The results presented suggest that the microfloras surrounding each mycorrhiza was influenced qualitatively by the type of mycorrhizal fungus present. The data reported in Tables 1 and 2 shows distinct differences in microbial populations and physiological activity of isolates from each microhabitat. These observations are similar to those reported by Katznelson, Rouatt, and Peterson (1962) for the rhizosphere microflora of birch seedling mycorrhizal rootlets.

Harley (1959) states that mycorrhizal infection of roots results in increased salt absorption and the mycorrhizal fungus exerts a selective influence upon kinds of salts or ions which may be absorbed by the plant root. Differences in numbers and types of organisms inhabiting the rhizosphere soil surrounding each mycorrhiza may be directly or indirectly attributed to the different fungal symbionts

infecting the rootlets. Part of this effect may be reflected in the availability or supply of inorganic and organic nutrients which could favor selective development of specific groups or species of microorganisms around each mycorrhizal type.

The data in Tables 2 and 3 show that, within the limitations of the experimental methods, a different kind of microflora exists around each root structure. This apparent influence of the mycorrhizae on associated microorganisms could possibly affect the susceptibility of the roots to invasion by pathogenic microbes. Lochhead (1940) has suggested resistance to a certain disease may be connected with microflora types favored by root excretions into the rhizosphere. Katznelson and Richardson (1948) found higher rhizosphere:nonrhizosphere microbial ratios where incidence of strawberry root-rot was decreased. It has been reported that metabolic activities of rhizosphere microorganisms are responsible for release of nutrients, growth substances, and complex organic metabolic products (Gawel, 1961; Gerretson, 1948; Katznelson, Lochhead, and Timonin, 1948).

As suggested by Katznelson, Rouatt, and Peterson (1962), the variance of types and kinds of bacteria surrounding the mycorrhizae may have a marked effect upon the nutrient uptake by mycorrhizal rootlets. In turn, this may induce more vigorous plant growth.

As may be inferred from the differences in ammonifying power and nitrate reducing capacity of the rhizosphere isolates from soil surrounding each mycorrhizal type (Table 3), the potential for liberation of ammonia from nitrogenous compounds as well as nitrate reductions may influence the amount and form of nitrogen available for assimilation by the tree roots. Ammonium nitrogen is readily utilized by many plants, whereas complex organic nitrogenous compounds must first be ammonified.

The percentage of Streptomyces based upon the total bacterial population was quite low compared to that of the suberized root rhizosphere; it was considerably lower with respect to nonrhizosphere soil (Table 1). The numbers and percentage of Streptomyces found in the rhizosphere soil compares favorably with results reported for similar forest soils (Wright and Bollen, 1961; Neal, Wright, and Bollen, 1965). Selective absorption of available nutrients or the production of antagonistic substances by the fungal symbionts or their associated rhizosphere microorganisms may account for the low proportion of Streptomyces found in the mycorrhizal rhizospheres. Even though the total Streptomyces population estimates were higher than those for nonrhizosphere soil, the low percentage of these higher bacteria in each mycorrhizal habitat would suggest that their activity also is quite low.

The results indicate that the rhizosphere populations of mycorrhizal roots differ according to morphological and physiological types in total numbers, not only in comparison with suberized root rhizosphere, and nonrhizosphere microfloras, but also with kind of mycorrhizal fungi.

Microfloras of one Douglas-fir Ectotrophic Mycorrhiza
and one Red Alder Ectotrophic Mycorrhiza

The rhizosphere microbial population estimates of a common dominant Douglas-fir mycorrhiza formed by C. graniforme and of adjacent suberized roots are shown in Table 4.

The rhizosphere microbial populations of C. graniforme mycorrhizal rootlets and adjacent suberized roots were considerably greater than those of nonrhizosphere soil (Table 4). The R/S ratios (rhizosphere microbial population/nonrhizosphere soil microbial population) were 3.3/1 and 4.3/1, respectively. The increase of microbial populations around the plant roots, compared to non-rhizosphere soil, is in agreement with other studies on a variety of plants (Rovira, 1965a). Usually the root tip supports a higher microbial population than other root portions. In contrast, the rhizosphere bacterial population of these particular mycorrhizal rootlets is lower than that of the suberized root.

Table 4. Microbial population estimates of rhizosphere and nonrhizosphere soil

Rhizosphere soil type	Bacteria	R/S ^a ratio	Molds	R/S ^a ratio
	^b millions		^b thousands	
Douglas-fir mycorrhizal root	91.6	3.3	347.3	2.0
Douglas-fir suberized root	120.0	4.3	300.0	1.7
Red alder mycorrhizal root	580.2	16.6	455.9	2.3
Red alder suberized root	300.7	8.6	275.0	1.4
Douglas-fir nonrhizosphere soil	28.0	---	175.0	---
Red alder nonrhizosphere soil	35.0	---	200.0	---

^aRhizosphere soil/nonrhizosphere soil ratio.

^bCounts per gram of soil, oven-dry basis. Each value represents a mean of three replicate samples.

Bacterial populations in red alder mycorrhizal and suberized root rhizospheres were considerably higher than in nonrhizosphere soil; the R/S ratios were 16.6 and 8.6, respectively. The bacterial populations in each rhizosphere of red alder were substantially higher than in those of Douglas-fir.

Mold populations were higher in the rhizosphere of mycorrhizal and suberized roots of Douglas-fir and red alder (Table 4) as compared to nonrhizosphere soil. Differences in mold populations of mycorrhiza and suberized root microhabitats of Douglas-fir were slight; the R/S ratios were 2.0 and 1.7, respectively. The mold population was, however, almost two times as great in the mycorrhizal rhizosphere as compared to the suberized root rhizosphere of red alder; the R/S ratios were 2.3 and 1.4, respectively. The mold population increase in the rhizosphere soil of the mycorrhizal rootlets agrees with results reported by Tribunskaya (1955), but not with those found by Katznelson, Rouatt, and Peterson (1962) and Neal, Bollen, and Zak (1964). Selective absorption or excretion of metabolites into the rhizosphere by the fungal symbiont or host plant may account for these differences. As with other studies (Katznelson, Rouatt, and Peterson, 1962; Neal, Bollen, and Zak, 1964), the predominant mold found in each mycorrhizal rhizosphere of each tree species was of the genus Penicillium.

The microbial populations of nonrhizosphere soil in the conifer and alder plots agree closely with those reported for the same soils by Chen (1965).

Members of the different physiological groups were more numerous in the rhizosphere of mycorrhizal and suberized roots of Douglas-fir and red alder than in comparable nonrhizosphere soils (Table 5). The most probable number of ammonifying and nitrate reducing microbes in rhizosphere soil of Douglas-fir mycorrhizal rootlets differed little from that of nonmycorrhizal, suberized roots. However, in rhizosphere of red alder mycorrhiza, more than three times the number of ammonifying than nitrate reducing microorganisms were observed. The opposite effect was found in the rhizosphere soil of suberized roots; the nitrate reducers were approximately three times greater than the number of ammonifying microorganisms. In all cases, the most probable number of ammonifiers and nitrate reducers were higher in each microhabitat as compared to nonrhizosphere soil.

Comparison of nonrhizosphere soils revealed that the Douglas-fir soil contained over twice as many ammonifying than nitrate reducing microbes. Red alder soil, however, harbored appreciably more nitrate reducers than ammonifiers.

The relative incidence of bacteria isolated from each rhizosphere and nonrhizosphere soil as related to their nutritional

Table 5. Most probable numbers of ammonifying and nitrate reducing microorganisms

Rhizosphere soil type	<u>Ammonifiers</u> ^a	<u>Nitrate reducers</u> ^b
	millions ^c	millions ^c
Douglas-fir mycorrhizal root	74.3	46.1
Douglas-fir suberized root	64.3	50.1
Red alder mycorrhizal root	128.8	47.3
Red alder suberized root	39.1	120.4
Douglas-fir nonrhizosphere soil	18.3	8.6
Red alder nonrhizosphere soil	17.5	25.3

^aProducing NH_4^+ in peptone water.

^bReducing NO_3^- in nitrate broth.

^cNumbers per gram of soil, oven-dry basis. Each value is the mean of the most probable number values for three replicates.

requirements, is shown in Table 6 for Douglas-fir and Table 7 for red alder.

Only 15% of the isolates from rhizosphere soil of Douglas-fir mycorrhizae were able to synthesize their requirements for growth from glucose-nitrate-salts media (Table 6). In contrast, 52% of the isolates required amino acids for growth. Only a small percentage of the isolates required growth factors and unidentified substances in yeast and soil extract for growth.

In comparison, 28% of the isolates from rhizosphere of suberized roots were able to satisfactorily grow in glucose-nitrate-salts media. Twenty-six percent required amino acids for growth. Only a low percentage of the isolates required complex substances.

On the contrary, 42% of the isolates from nonrhizosphere soil required complex unidentified substances in soil extract for growth and 21% required a combination of the substances in yeast and soil extracts.

Thirty-eight percent of the bacteria isolated from red alder mycorrhizal rhizosphere soil needed amino acids in order to grow and 26% needed growth factors (Table 7). Yeast or soil extract was required by only a low percentage of the isolates.

As was noted for the isolates from rhizosphere soil of Douglas-fir suberized roots, the majority of the microbes isolated from the red alder suberized root rhizosphere could synthesize their

Table 6. Frequency (in %) of bacterial nutritional groups isolated from the rhizosphere of Douglas-fir mycorrhizal rootlets, suberized roots, and nonrhizosphere soil

Nutritional group	Rhizosphere soil type		Nonrhizosphere soil
	Mycorrhizal rootlets	Suberized roots	
B (minerals, glucose)	15	28	3
A (B + Casamino acids)	52	26	2
G (B + growth factors)	13	17	5
AG (B + a. a. + g. f.)	12	10	7
Y (B + yeast extract)	5	6	12
S (B + soil extract)	6	9	42
YS (B + y. e. + s. e.)	0	4	21
No growth in test media	2	0	8

Table 7. Frequency (in %) of bacterial nutritional groups isolated from the rhizosphere of red alder mycorrhizal rootlets, suberized roots, and nonrhizosphere soil

Nutritional group	Rhizosphere soil type		Nonrhizosphere soil
	Mycorrhizal rootlets	Suberized roots	
B (minerals, glucose)	5	36	3
A (B + Casamino acids)	38	24	5
G (B + growth factors)	26	7	11
AG (B + a. a. + g. f.)	15	8	12
Y (B + yeast extract)	6	9	2
S (B + soil extract)	4	6	38
YS (B + y. e. + s. e.)	4	7	20
No growth in test media	2	3	9

requirements for growth from glucose-nitrite-salts medium or required one or more amino acids for growth. Similarly, only a low percentage of the isolates required yeast or soil extracts for growth.

A large percentage of the isolates from red alder nonrhizosphere soil required soil extract or a combination of substances found in soil and yeast extracts for growth. These nutritional groupings of isolates are similar to those found for microbes isolated from Douglas-fir nonrhizosphere soil (Table 6).

Metabolic Activity in Simulated Rhizospheres

The influence of Douglas-fir and red alder root suspensions on metabolic activity of nonrhizosphere microbes is shown in Figures 8 through 11. Results are expressed as microliters of oxygen uptake beyond that of endogenous (soil only) values. This study was an attempt to simulate an artificial rhizosphere in a Warburg flask by modification of the sterile sand and collodion membrane techniques (Rovira, 1956 and Timonin, 1941).

The metabolic activity of the soil microflora was stimulated by a slurry of Douglas-fir nonmycorrhizal, suberized roots (Figure 8). After 12 hours, oxygen uptake was 1.5 times as great as nonrhizosphere soil plus glucose. Combining the root slurry with glucose resulted in a substantial increase in microbial activity as shown by increased oxygen consumption; the increase being approximately

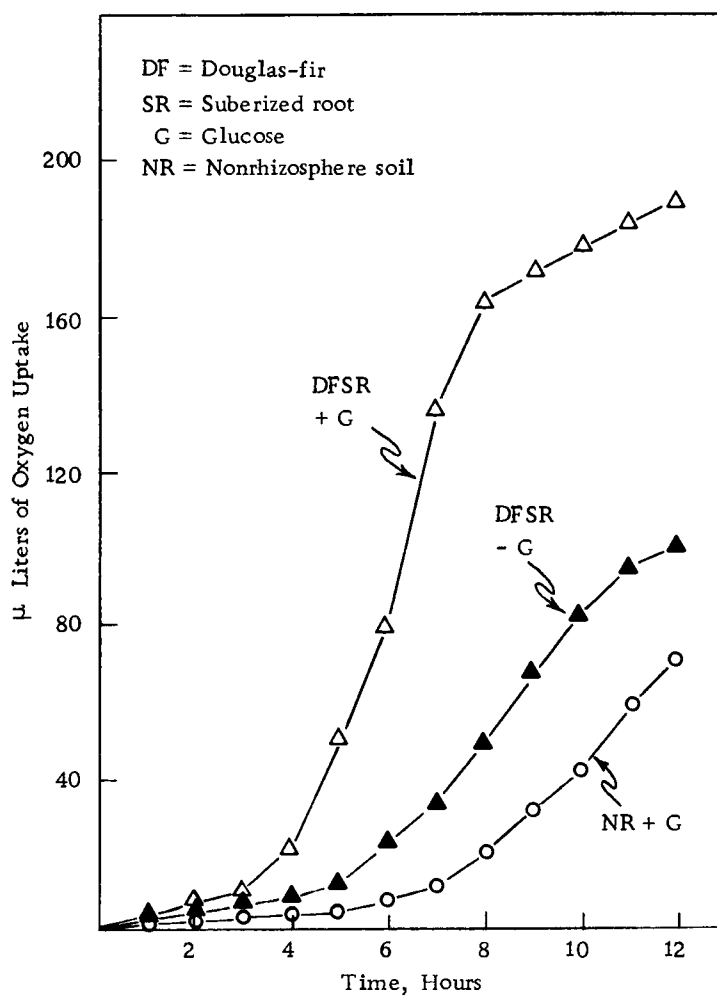


Figure 8. Microbial oxidation of Douglas-fir nonmycorrhizal suberized root slurry with and without glucose (microliters of oxygen uptake beyond that of endogenous (soil only) values).

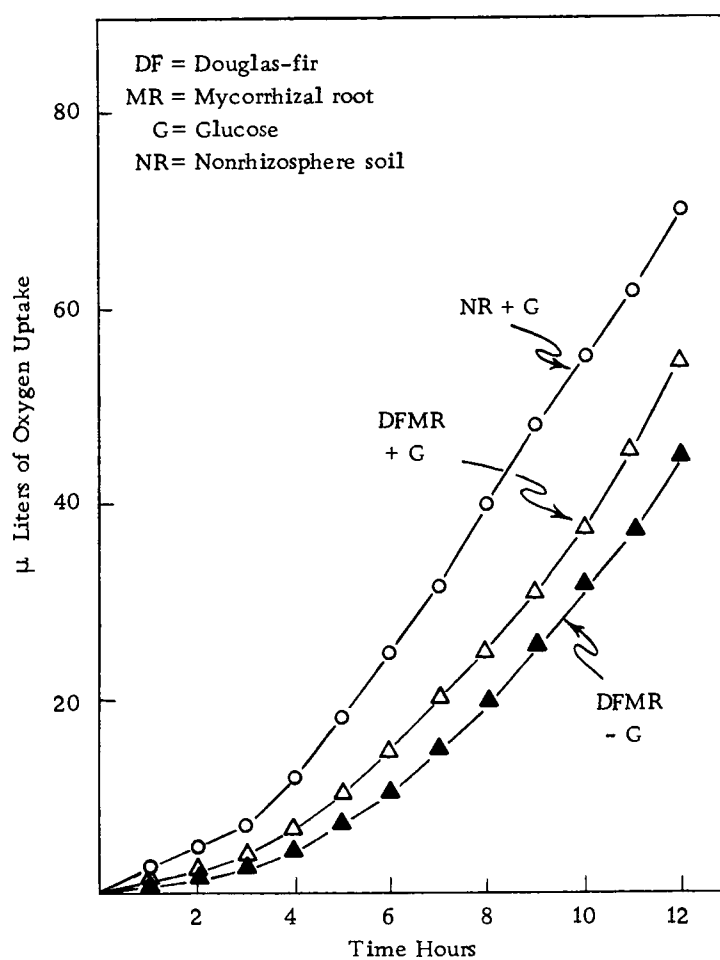


Figure 9. Microbial oxidation of Douglas-fir mycorrhizal root (*Cenococcum graniforme*) slurry with and without glucose (as in Figure 8).

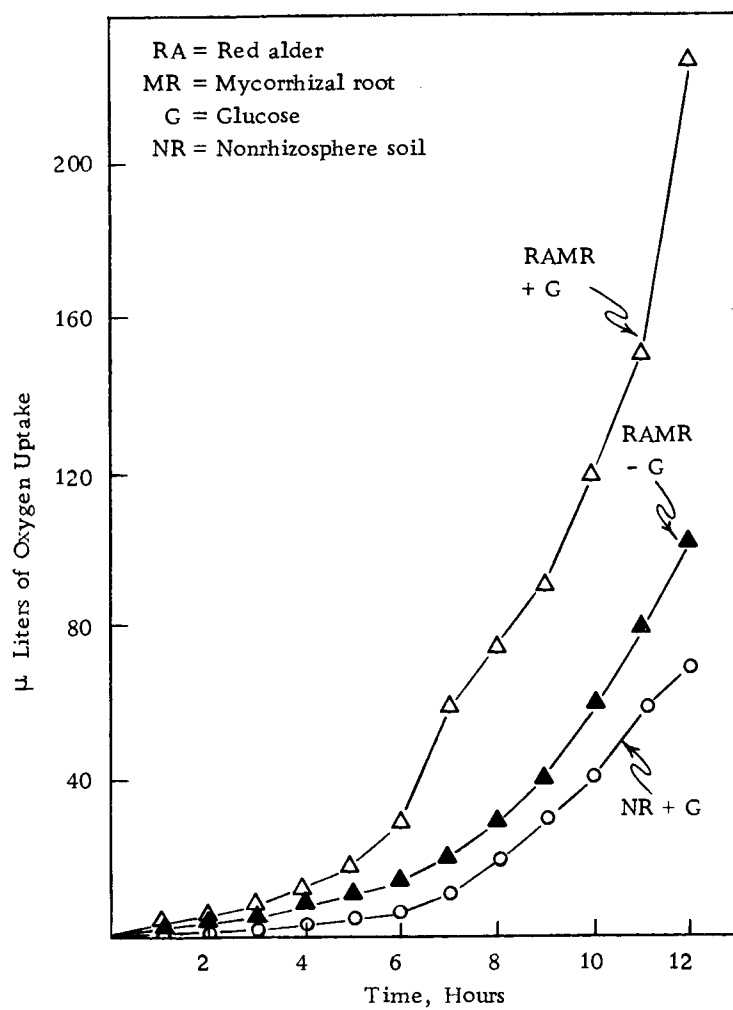


Figure 10. Microbial oxidation of red alder mycorrhizal root slurry with and without glucose (as in Figure 8).

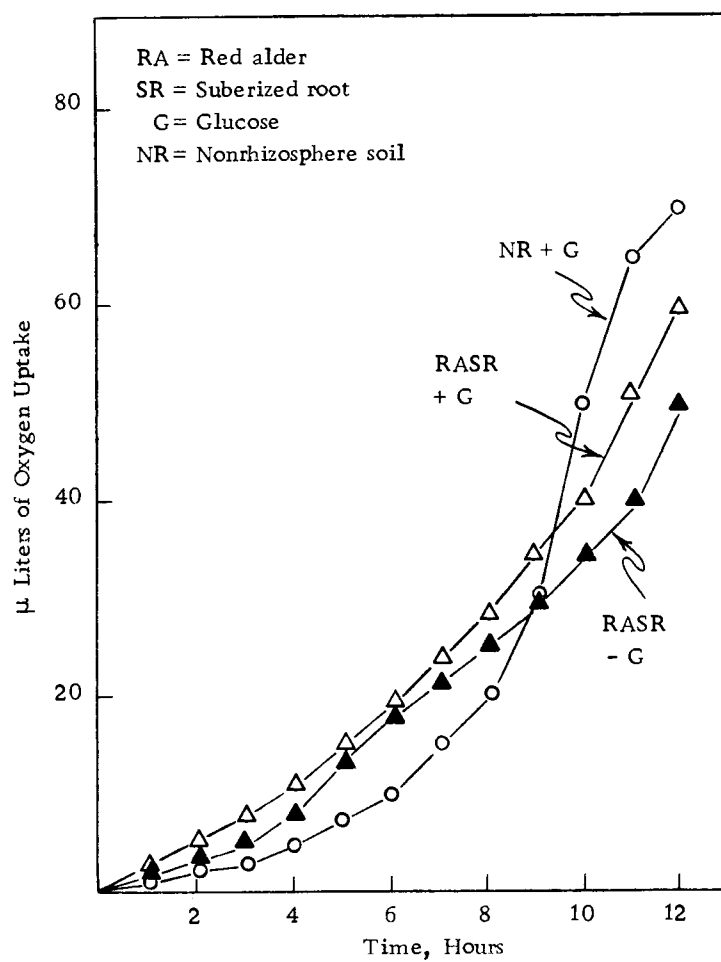


Figure 11. Microbial oxidation of red alder nonmycorrhizal suberized root slurry with and without glucose (as in Figure 8).

2.5 times greater than the oxidation rate of nonrhizosphere soil plus glucose.

The opposite effect was produced by a slurry of Douglas-fir mycorrhizae formed by C. graniforme, an antibiotic producing fungus (Krywolap and Casida, 1964; Krywolap, Grand, and Casida, 1964). As shown in Figure 9, the oxidation rate was appreciably less than for nonrhizosphere soil plus glucose. Adding glucose with the mycorrhizal root slurry caused little increase in oxygen uptake; oxygen consumed was still less than for nonrhizosphere soil plus glucose. The data suggest a selective inhibitory phenomenon perhaps due to influence of the antibiotic produced by the fungus.

Soil microbial activity was stimulated by red alder mycorrhizal root suspension as compared to nonrhizosphere soil plus glucose (Figure 10). Incorporation of glucose with the red alder mycorrhizal root slurry resulted in oxygen uptake values approximately 3.5 times as great as those of nonrhizosphere soil plus glucose.

The influence of red alder nonmycorrhizal, suberized root slurry on the oxidative respiration patterns of soil microbes is shown in Figure 11. The microbial oxidation rate was considerably less in the presence of red alder suberized root slurry than with nonrhizosphere soil plus glucose. Adding glucose with the suberized

root slurry caused little additional stimulation of microbial metabolic activity.

These oxidative respiratory patterns are similar to those obtained for Douglas-fir mycorrhizal rootlets formed by C. graniforme, suggesting an inhibitory phenomenon here as well. To explore this possibility, an antibacterial substance in red alder root and nodule slurries was sought by the seeded plate--paper disk technique. Disks saturated with slurries prepared from summer collections effectively inhibited Bacillus cereus and B. subtilis on glucose salts agar, but not on peptone-beef extract agar (Figure 12). Slurries prepared from winter collections after leaf fall were not inhibitory on either medium. Likewise, no inhibition on either medium was observed with red alder mycorrhizal root slurries.

Data for the Douglas-fir and red alder mycorrhizae suggest that the microflora surrounding these mycorrhizal rootlets are influenced qualitatively and quantitatively by the fungal symbiont present. The distinct differences in bacterial and mold populations (Table 4) can be attributed to the excretion of inhibitory or stimulatory substances by the fungal symbiont, host plant, or both, into the rhizosphere thus favoring a selective development of microorganisms in each microhabitat. For example, resistance to certain root diseases by plants has been suggested to be related to microfloral types favored by root exudates (Lochhead, 1940, Lochhead, Timonin

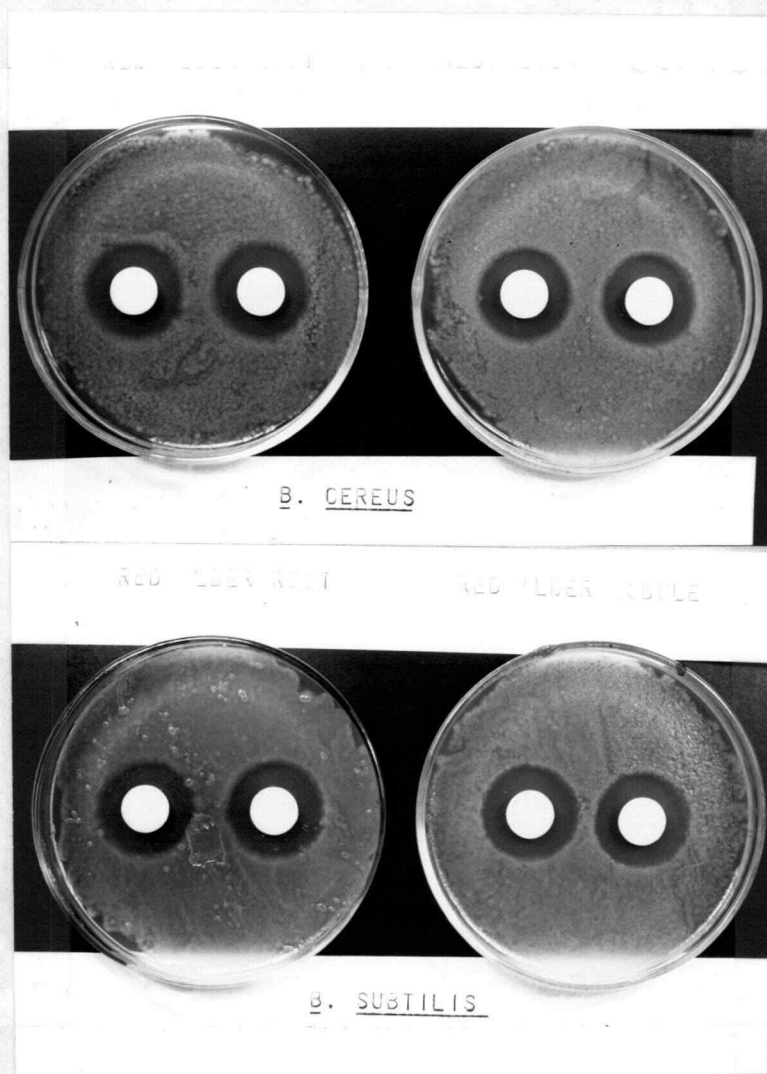


Figure 12. Growth inhibition of Bacillus cereus and B. subtilis by red alder root and nodule extracts on glucose-salts medium.

and West, 1940). Likewise, Timonin (1966) reported that the rhizosphere microflora of diseased lodgepole pine seedlings differed considerably from the rhizosphere of healthy lodgepole pine seedlings. A high number of spore-forming bacilli capable of producing substances inhibitory towards Fusarium and Rhizoctonia species were present in the rhizosphere of normal seedlings, but not in that of the diseased seedlings. The prevalence of these microbes in the rhizosphere of healthy seedlings was attributed to preferential secretions from the pine roots.

It is of interest to note in this respect that the rhizosphere bacterial population of Douglas-fir mycorrhiza formed by C. graniforme was less than that of the suberized root rhizosphere. The opposite effect was reported in previous studies in which all bacterial populations of the rhizosphere soil surrounding mycorrhizal rootlets were higher (Neal, Bollen, and Zak, 1964; Katznelson, Rouatt, Peterson, 1962; Tribunskaya, 1955). A non-water-soluble antibiotic has been reported to be produced by C. graniforme in pure culture (Krywolap and Casida, 1964) and in nature (Krywolap, Grand, and Casida, 1964). Even in low concentrations the antibiotic effectively inhibited growth of gram positive bacteria, and in high concentrations, gram negative bacteria, yeasts, and Streptomyces. However, there was no inhibition of the several fungi investigated. In the present study, the lower bacterial populations in the rhizosphere of Douglas-fir mycorrhizae

formed by C. graniforme may be due to the selective inhibitory action of an antibiotic produced by the fungal symbiont. Selective absorption of assimilation of organic or inorganic nutrients may also influence the rhizosphere quantitatively.

In contrast, the rhizosphere microflora of red alder mycorrhiza appears to be stimulated by the fungal symbiont, as is evident from the higher microbial numbers found in this microhabitat.

The data in Table 5 show the existence of different physiological groups which are stimulated in each microhabitat and non-rhizosphere soil. These observations are similar to those of Katznelson, Rouatt, and Peterson (1962) and Neal, Bollen, and Zak (1964). The greater numbers of these groups in rhizosphere as compared to nonrhizosphere soil suggests that in the rhizosphere there are more nutrients capable of being ammonified, nitrified, and subsequently denitrified. A synthetic or solubilizing action of the mycorrhizal fungus may directly or indirectly render more nutrients available to the rhizosphere microflora. Numerical differences in relation to physiological activity are not great between Douglas-fir mycorrhizal and nonmycorrhizal rhizosphere soils, but appreciably different physiological groups do exist between the rhizosphere of mycorrhizal and suberized roots of red alder.

As may be inferred from the differences in most probable numbers of ammonifying and nitrate reducing microbes in each

microhabitat, the potential for liberation of ammonia from proteins, amino acids, and complex nitrogenous compounds may markedly affect the availability of nitrogen to the tree. For example, a more rapid breakdown of amino acids and simple proteins to ammonia nitrogen has been reported by Katznelson and Rouatt (1957) to occur in rhizosphere than in nonrhizosphere soil. Changes in acidity near bean roots has been observed to alter ammonium assimilation (Barker, Volk, and Jackson, 1966).

The selective action of the mycorrhizae upon the rhizosphere microflora as related to nutritional requirements is shown in Tables 6 and 7.

A large percentage of the isolates from the rhizosphere of the Douglas-fir mycorrhiza required one or more amino acids for growth while relative lower proportions of the isolates required yeast and soil extract for growth. In comparison, 28% of the microbes isolated from the suberized root rhizosphere were able to synthesize their growth requirements from the glucose-salts agar; an increase of 13% as compared to the isolates from the mycorrhizal rhizosphere. A large proportion of the suberized root isolates also required amino acids for growth, while a relatively low proportion of the isolates required yeast and soil extracts for growth. In contrast, 42% of the nonrhizosphere isolates required

unidentified substances in soil extract and 21% needed a combination of one or more substances in yeast and soil extracts.

A similar pattern occurred with isolates from the red alder rhizosphere and nonrhizosphere soil (Table 7). Rhizosphere isolates from the soil surrounding mycorrhizal rootlets required amino acids, growth factors, or a combination of amino acids and growth factors, while a large proportion of the isolates from the suberized root rhizosphere were able to synthesize these growth requirements in the glucose-salts medium or from media supplemented with amino acids. Likewise, isolates from nonrhizosphere soils required unidentified substances in soil and yeast extracts. These observations, based upon substrate utilization, indicate that the rhizosphere microbes of Douglas-fir and red alder roots are less fastidious than the nonrhizosphere microflora. Similarly, the microbes associated with suberized root rhizospheres of Douglas-fir and red alder appear to require less complex nutrients for growth than do those found in the rhizosphere of mycorrhizal rootlets.

The apparent influence of the mycorrhizae on associated microorganisms, through selective stimulation or inhibition of certain microbial groups, could possibly affect the susceptibility of roots to invasion by plant pathogenic microorganisms. As suggested by Neal, Bollen, and Zak (1964) and Zak (1964), the metabolic products of these groups may alter the environment of the root microhabitat

by changing its acidity, or by selective assimilation or synthesis of nutrients, and thus form an effective biological barrier to root pathogens.

Root exudates and decaying root material or sloughed off cells are generally believed to exert the greatest influence on microorganisms in the rhizosphere (Rovira, 1965a). Healthy plants, and probably diseased plants also, exude a wide variety of amino acids, organic acids, growth factors, and enzymes (Rovira, 1965b). However, the amounts of exudate and cellular debris available to rhizosphere microorganisms as an energy source at any one time are not known.

An attempt was made to measure the influence of mycorrhizal and nonmycorrhizal exudate and cellular material on microbial activity by making available a proportional aliquot of this material to nonrhizosphere microbes and measuring their metabolic responses manometrically. The rhizosphere microbes are, for all practical purposes, nonrhizosphere microorganisms which were selectively stimulated and then proliferated in the root microhabitat. Although the seedcoat microflora of Douglas-fir and red alder may play a role in the early initiation of the rhizosphere effect, it is doubtful they are of any significance after root growth and infection by mycorrhizal fungi.

The metabolic activity of the soil microorganisms was stimulated in all experiments by Douglas-fir and red alder mycorrhizal and suberized root slurries as compared to soil only (endogenous) oxidative respiration (Figures 8-11). This is in agreement with the other manometric studies of rhizosphere microbes (Zagallo and Katznelson, 1957; Zagallo and Bollen, 1962).

In particular, activity of the nonrhizosphere microflora differed in relation to metabolism of glucose. Metabolic activity was appreciably stimulated by addition of glucose to the Warburg flask containing Douglas-fir suberized root material (Figure 8), but when incorporated with Douglas-fir mycorrhizal material (Figure 9), little oxidative respiration increase was observed. If more than one substrate is available as a carbon source, all other factors being equal, microorganisms will utilize the carbon source which requires the least amount of energy to degrade and assimilate. Glucose is easily utilized with a minimum of energy expenditure by most microorganisms and thus in most cases is preferentially utilized by microbial cells. Based upon this analogy, the oxidation rates with mycorrhizae plus glucose would be expected to be at least as great as with soil only plus glucose or perhaps higher as was with Douglas-fir suberized roots (Figure 8). This not being the case, however, a selective inhibition of microbial oxidation is indicated, presumably due to the antibiotic produced by the fungal symbiont, C. graniforme

(Krywolap and Casida 1964; Krywolap, Grand and Casida 1964).

Although the results are not conclusive, it appears that the antibiotic effectively inhibited the utilization of glucose as an energy source by certain groups of bacteria. Even though the antibiotic is not water soluble, the results indicate it has an active influence in the rhizosphere (Figure 9, Tables 4, 5). The mycorrhizal fungus thus is able to directly influence the rhizosphere microflora by its metabolic products and selectively stimulate or inhibit specific microbial groups.

The red alder mycorrhizal root slurry stimulated considerably the metabolic activity of nonrhizosphere microbes (Figure 10). Incorporating glucose brought about an even greater response. The results indicate that the mycorrhizal root material is highly stimulative, and are in agreement with data presented in Tables 1 and 2.

A metabolic oxidation rate pattern similar to that obtained with mycorrhizal roots of Douglas-fir formed by C. graniforme also was observed with red alder root material (Figure 11). Incorporation of glucose with the root slurry did not cause an appreciable increase in metabolic activity, indicating the presence of an inhibitory substance, particularly with reference to glucose oxidation. These results are similar to those of Basaraba (1966) who investigated manometrically the influence of oxidized polyhydroxy phenols from chestnut wood and wattle bark on the metabolic activity of selected pure cultures of

bacteria and found glucose oxidation by Azotobacter chroococcum to be completely inhibited in the presence of 0.5% (W/V) polyphenolic compounds. Moreover, endogenous respiration of Azotobacter vinelandii and Escherichia coli was reduced considerably in the presence of polyphenolic compounds with Rhizobium meliloti, Rhizobium sp., Saccharomyces cerevisiae and a Rhodotorula species; Pseudomonas fluorescens, however, was stimulated.

A biological assay for antagonistic materials in red alder spring and summer roots and nodules (Figure 12) revealed a substance which effectively inhibited the growth of Bacillus cereus and B. subtilis on glucose-salts media, but not on peptone-beef extract agar. Extracts of root and nodule materials collected during winter months did not contain substances inhibitory to the bacilli.

The identity or action of this substance or substances from red alder is not known, but its nature probably lies in one or more of three areas. First, oxidized polyphenols have been implicated as defense mechanisms against root attacking microbes (Hare 1966). The methods of preparing the root and nodule extracts may have triggered the oxidation of phenolic materials by polyphenol oxidases to biologically toxic quinones. These phytotoxic compounds may have been responsible for the growth inhibition of the bacilli observed on glucose-salts medium. Li, Lu, Trappe and Bollen (1967) have demonstrated polyphenol oxidase activity in leaf extracts of red

alder; no activity was observed with Douglas-fir needle extracts. Inhibition of glucose oxidation by the red alder suberized root material (Figure 11) and uninhibited growth of the bacilli on peptone-beef extract agar supports this possibility. These phytotoxic compounds may not ordinarily be active in the rhizosphere, however, since polyphenol oxidase is usually not activated until root tissues are injured (Hare, 1966). Our data show that the red alder suberized root microhabitat fosters a highly active rhizosphere microflora (Table 1 and 2).

Secondly, an antibiotic produced by the red alder nodule endophyte may be present in the root and nodule extracts and effectively inhibit the growth of bacilli (Figure 12). Based upon studies of other species of Alnus (Uemura 1952a, Uemura 1952b, and Mikola 1965), the nitrogen-fixing endophyte of red alder nodules is presumably a Streptomyces species. Mikola (1965) found that several Streptomyces species isolated from Alnus glutinosa nodules produced antibiotics which effectively inhibited growth of Fomes annosus, a root pathogen. The strongest antagonism was exerted by strains capable of forming nodules with the host plant in controlled inoculation experiments. Considine and Casida (1964) observed that certain species of Streptomyces could grow and produce antibiotics on nitrogen-deficient media.

Cytological observations have shown the Alnus glutinosa endophyte, which appear similar to that of Alnus rubra (Figure 13) in cross section, changes morphologically from an active stage in the summer to a resting stage after leaf fall during the winter months (Gardner, 1965). This could explain the lack of inhibitory substances in root and nodule extracts obtained from material collected during the winter months. The influence of an antibiotic upon the rhizosphere microflora may be negligible since its action would depend upon its concentration in the rhizosphere. Bacteria are well known for their ability to mutate to antibiotic resistant strains, especially if the antibiotic is present in sub-lethal concentrations.

If both the production of an antibiotic and oxidation of phenolic compounds to quinone should be active at the same time, a synergistic action may result. Vörös, Kiraly and Farbas (1957) have reported that in vitro streptomycin is ineffective against Phytophthora but when absorbed through the roots, the antibiotic confers resistance to the plants and enhances polyphenol oxidase activity. If this is the case, lack of either antibiotic production or polyphenol oxidase synthesis may be the reason why no inhibitory substances were found in extracts of root and nodule material during the winter months.

These results indicate that the rhizosphere microbes of Douglas-fir and red alder are influenced qualitatively and quantitatively by the mycorrhizal fungus present. Metabolic secretions of

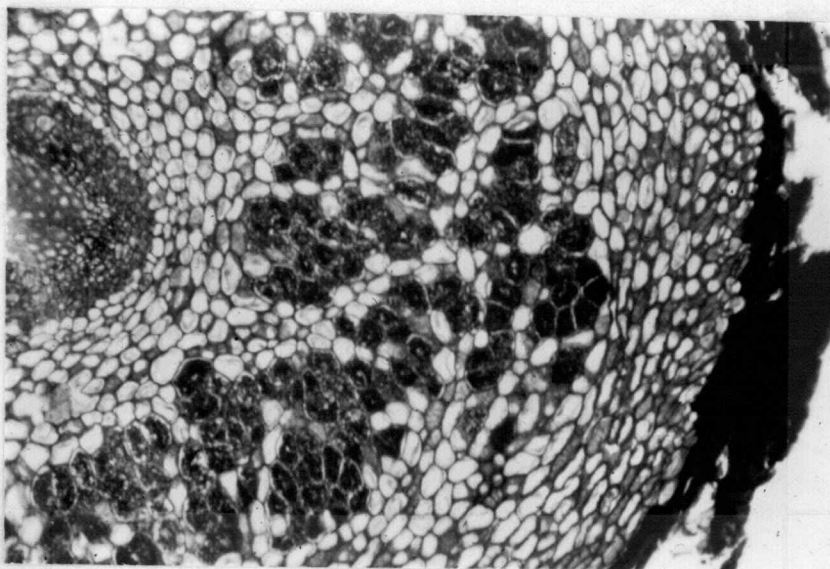


Figure 13. Cross-section of red alder nodule (x 150). The dark stained material of the inner tiers of cortical cells is the nitrogen-fixing endophyte, presumably a Streptomyces.

the mycorrhizal fungus and associated suberized roots probably play an important role in influencing the kind and type of microorganisms that are to be found in each microhabitat. The importance of these excreted metabolites is not fully known, but they could be important in discouraging attack of roots by parasites by favoring the development of specific rhizosphere microbes which form a biological barrier.

SUMMARY

Extensive morphological investigations of the predominating Douglas-fir and red alder ectotrophic mycorrhizae found on rootlets collected at the Cascade Head Experimental Forest near the northern Oregon Coast were conducted.

Jet-black mycorrhizae predominated on Douglas-fir rootlets collected from a mixed conifer stand lacking alder. Gross and cross-sectional morphological characteristics of these mycorrhizae were compared to those described by Trappe (1962) and the fungal symbiont was identified Cenococcum graniforme.

Red alder rootlets collected from pure stands adjacent to the conifers were examined and revealed that two mycorrhizae predominated. Gross and cross-sectional morphological studies of the mycorrhizal roots, the first for this tree species, were conducted. One ectotrophic mycorrhizae form was found to occur on 40 percent of the rootlets collected. The mycorrhiza was clavate with a dark-brown roughened fungal mantle. The Hartig net was well defined and penetrated between the outer tier of root cortical cells. Septate hyphae with clamp connections sometimes originated from turgid cells at the mantle surface. Based on these observations, the fungal symbiont was classed as a Basidiomycete. Another common form of mycorrhiza, found on about 50 percent of the roots collected, was

pale brown and glabrous. No mycorrhizal mantle was obvious under low-power magnification. A single-layered Hartig net sporadically penetrated the rootlet epidermis. The mycorrhiza was considered weakly ectotrophic.

The rhizosphere microflora of three morphologically different mycorrhiza of a single Douglas-fir were examined and compared to rhizosphere microflora of adjacent suberized roots and to non-rhizosphere microflora. Populations surrounding mycorrhizal formations were significantly greater than those of nonrhizosphere soil. Likewise, significantly large differences were found between the rhizosphere bacterial populations of the different mycorrhizal formations as compared to each other and the adjacent suberized roots. Mold populations in each rhizosphere were less than those of nonrhizosphere soil. Members of the genus Penicillium predominated in the rhizosphere soil.

Different morphological and physiological types were found to predominate in each rhizosphere and nonrhizosphere soil. The distinct differences found in each mycorrhizal rhizosphere as compared to each other and to nonrhizosphere soil was attributed to influence of the fungal symbiont present.

A similar investigation of the rhizosphere microfloras of Douglas-fir Cenococcum graniforme mycorrhizae and one type of ectotrophic mycorrhizae of red alder, and of nonmycorrhizal,

suberized roots of both tree species was conducted. Distinct differences in microbial populations and most probable numbers of ammonifying and nitrate-reducing microbes were found in each microhabitat and nonrhizosphere soil.

Nutritional classification of isolates from each rhizosphere microhabitat and nonrhizosphere soil revealed that most of the microbes from rhizosphere soil surrounding mycorrhizal or suberized roots were able to synthesize their growth requirements from glucose-salts medium, or that they required one or more amino acids for growth. In contrast, a high percentage of the isolates from nonrhizosphere soils required complex growth factors in yeast and/or soil extract for growth.

Warburg studies showed that homogenized Douglas-fir non-mycorrhizal suberized root and red alder mycorrhizal root suspensions highly stimulated respiration of soil microbes, especially in the presence of glucose. Glucose oxidation was, however, suppressed in the presence of Douglas-fir mycorrhizal root suspension. This lack of metabolic stimulation was attributed to the presence of an antibiotic which is reported to be produced by the fungal symbiont, Cenococcum graniforme. The metabolic oxidative pattern was similar in the presence of red alder nonmycorrhizal root suspension plus glucose. An antibacterial substance was found to be present in the red alder root suspension and also in red alder

nodule suspension. The antagonistic substance effectively inhibited growth of Bacillus subtilis and B. cereus on glucose-salts agar.

The influence of the mycorrhizae and suberized roots of each tree species on the rhizosphere microfloras was attributed to secretion of inhibitory or stimulatory substances by the fungal symbiont, host plant, or both, into the rhizosphere.

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