A comparison of rhizosphere microfloras associated with mycorrhizae of red alder and Douglas-fir

Abstract

Rhizosphere microfloras of Cenococcum graniforme (Sow.) Ferd. and Winge mycorrhiza of Douglas-fir (Pseudotsuga menziesii (Mirb.) Franco), of one type of ectotrophic mycorrhiza of red alder (Alnus rubra Bong.), 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 between rhizosphere microhabitats. 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, C. graniforme, is reported to produce. Glucose oxidation by nonrhizosphere microbes was similarly repressed in the presence of red alder nonmycorrhizal root suspension. An antagonistic substance found in red alder root and nodule suspensions inhibited growth of Bacillus subtilis (Cohn) Przamowski and B. cereus Frankland and Frankland on glucose-salts agar. These experimental results are discussed with reference to the influence of mycorrhizal and adjacent nonmycorrhizal suberized roots upon rhizosphere microfloras.

Introduction

The term “rhizosphere” was first introduced by Hiltner in 1904 to designate the region of soil immediately influenced by plant roots. Numerous investigators have since reported higher microbial activity and populations in the rhizosphere of many plants than in adjacent soil (Katznelson et al., 1948; Clark, 1949; Starkey, 1958; Rovira, 1965; and Timonin, 1965). However, little has been published on the influence of mycorrhizae on

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rhizosphere microfloras. Tribunskaya (1955) compared the rhizosphere microfloras of 5-month-old pine seedlings lacking or having poorly developed mycorrhizae with those of similarly aged seedlings with well-developed mycorrhizae. She found considerably higher populations of fungi and proteolytic and fluorescent bacteria in rhizosphere soil of mycorrhizae than that of nonmycorrhizal roots. Katznelson et al. (1962) observed higher bacterial and actinomycete populations but lower numbers of fungi in the rhizosphere of yellow birch mycorrhizae than in that of nonmycorrhizal roots. The influence of mycorrhizal fungi upon the rhizosphere of a single Douglas-fir tree was demonstrated by Neal et al. (1964) who compared (1) three morphologically distinct mycorrhizae, sometimes side by side on the same rootlet, (2) adjacent suberized roots, and (3) nonrhizosphere soil. Each mycorrhizal rhizosphere differed significantly from the others in populations of bacteria, fungi, and *Streptomyces*, the difference being attributed to the type of fungal symbiont present. The suberized roots and nonrhizosphere soil differed from the mycorrhizae as well as from each other.

The present study was initiated as part of a broad investigation of soil microbes in relation to biological control of tree root disease. Red alder was included in the study because preliminary investigation suggested its possible role in inhibiting certain root pathogens (Li et al., 1968) and because of its ecological significance in forests of the Pacific Northwest as a nonleguminous, nitrogen-fixing tree (Tarrant, 1961; Chen, 1965).

**Materials and Methods**

Ectotrophic mycorrhizae, suberized roots, and adjacent nonrhizosphere soil were collected from the upper 8 inches of soil in a pure stand of red alder and, for Douglas-fir, from a mixed-conifer stand lacking alder at the Cascade Head Experimental Forest near the northern Oregon coast. Samples were stored at 1°C and processed within 24 hours after collection.

Examination of root collections revealed that each tree species had an abundance of distinctive, identifiable, ectotrophic mycorrhizae. For Douglas-fir, the distinctive mycorrhizal type was formed with *Cenococcum graminiforme* (Sow.) Ferd. and Winge, a fungus that commonly forms mycorrhizae on Douglas-fir throughout the region (Trappe, 1962). For alder, the distinctive mycorrhiza was dark brown and rough, formed by an unidentified basidiomycete (Neal et al., 1968). These two types were used in our experiments. Nonmycorrhizal suberized roots were taken from the same collections in lieu of nonmycorrhizal root tips, which could not be found. Three 1-kg portions of nonrhizosphere soil were collected from near the sampled roots at each sampling location.

Samples were taken from three locations in each stand, each sample being analyzed independently as a replicate.

Rhizosphere and nonrhizosphere microbial populations were estimated by the dilution pour-plate method on soil extract agar (Allen, 1957) for bacteria, and on Martin's (1950) rose bengal agar for molds, as described by Neal et al.
(1964), Neal et al. (1966), and Neal et al. (1968). The most probable number of microbes capable of producing ammonia from peptone water and those reducing nitrate to nitrite in 1-percent KNO₃ broth were determined according to the method of Alexander (1965). After 7 days' incubation at 28 C, ammonia production from peptone water was detected by Nessler's reagent, and the presence of nitrites, indicating the reduction of nitrate in 1-percent KNO₃ broth, was determined by Griess' reagent (Pelczar, 1957).

In metabolic studies, respiration was measured by the direct method (Umbreit et al., 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-percent sodium hypochlorite solution for 5 min, and serially washed in 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 (molar) 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 non-rhizosphere 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 as inoculum to each Warburg flask, which also 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-percent 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. 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 solutions previously pipetted in the flask sidearm was tipped in immediately after equilibration.

Assays for biologically antagonistic substances in red alder roots and nodules were determined by the paper-disk plate method (Loo et al., 1945) with stock cultures of *Bacillus cereus* Frankland and Frankland and *B. subtilis* (Cohn) Prazmowski. Root and nodule slurries were prepared as previously described from material collected during the summer and winter months and concentrated approximately fiftyfold by lyophilization. Paper disks were impregnated with the slurry suspensions, placed on peptone-beef extract and glucose-salts agar seeded with the test organisms, and incubated for 48 hr at 30 C. Controls were paper disks impregnated with sterile distilled water.

**Results**

**BACTERIAL POPULATIONS**

Rhizosphere microbial populations of mycorrhizae formed by *C. graminiforme* and of adjacent suberized roots of Douglas-fir were greater than those
of nonrhizosphere soil (Table 1). The R/S ratios (rhizosphere microbial population/nonrhizosphere soil microbial population) were 3.3/1 and 4.3/1, respectively, agreeing with results of other studies on a variety of plants (Rovira, 1965).

Bacterial populations in red alder mycorrhizal and suberized root rhizospheres were substantially higher than nonrhizosphere soil; the R/S ratios were 16.6 and 8.6, respectively.

TABLE 1. Microbial population estimates of rhizosphere and nonrhizosphere soil

<table>
<thead>
<tr>
<th>Rhizosphere soil type</th>
<th>Bacteria</th>
<th>R/S ratio(^1)</th>
<th>Molds</th>
<th>R/S ratio(^1)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Millions(^2)</td>
<td>Thousands(^2)</td>
<td></td>
</tr>
<tr>
<td>Douglas-fir mycorrhizal root</td>
<td>91.6</td>
<td>3.3</td>
<td>347.3</td>
<td>2.0</td>
</tr>
<tr>
<td>Douglas-fir suberized root</td>
<td>120.0</td>
<td>4.3</td>
<td>300.0</td>
<td>1.7</td>
</tr>
<tr>
<td>Red alder mycorrhizal root</td>
<td>580.2</td>
<td>16.6</td>
<td>455.9</td>
<td>2.3</td>
</tr>
<tr>
<td>Red alder suberized root</td>
<td>300.7</td>
<td>8.6</td>
<td>275.0</td>
<td>1.4</td>
</tr>
<tr>
<td>Douglas-fir nonrhizosphere soil</td>
<td>28.0</td>
<td>–</td>
<td>175.0</td>
<td>–</td>
</tr>
<tr>
<td>Red alder nonrhizosphere soil</td>
<td>35.0</td>
<td>–</td>
<td>200.0</td>
<td>–</td>
</tr>
</tbody>
</table>

\(^1\) Rhizosphere/nonrhizosphere soil ratio.
\(^2\) Counts per gram of soil, oven-dry basis. Each value represents a mean of three replicate samples.

MOLD POPULATIONS

Mold populations were higher in the rhizosphere of mycorrhizal and suberized roots of Douglas-fir and red alder (Table 1) as compared with nonrhizosphere soil. Differences between mycorrhizal and suberized-root microhabitats of Douglas-fir were slight (R/S 2.0 and 1.7, respectively). For red alder, however, mold populations were more than 1-1/2 times as great in the mycorrhizal rhizosphere as in the suberized root rhizosphere (R/S 2.3 and 1.4, respectively). The rhizosphere mold population increases agree generally with those reported by Tribunskaya (1955) but not with those found by Katznelson et al. (1962) and Neal et al. (1964).

PHYSIOLOGICAL GROUPS

Members of the different physiological groups were much more numerous in the rhizospheres of mycorrhizae and suberized roots of Douglas-fir and
red alder than in nonrhizosphere soils (Table 2). The most probable number of ammonifying and nitrate-reducing microbes in the rhizosphere of Douglas-fir mycorrhizae differed little from that of nonmycorrhizal suberized roots. However, in rhizospheres of red alder mycorrhizae, more than three times the number of ammonifying than nitrate-reducing microorganisms were observed. The opposite was found in the rhizosphere soil of suberized roots; the nitrate reducers were approximately three times greater than the number of ammonifying microorganisms.

**METABOLIC ACTIVITY**

The influence of Douglas-fir and red alder root suspensions on metabolic activity of nonrhizosphere microbes is shown in Figures 1 through 4. Results are expressed as microliters of oxygen uptake beyond that of endogenous respiration (soil only). A successful attempt was made in the studies to simulate an artificial rhizosphere in a Warburg flask by modifications of the sterile sand and collodion membrane techniques (Rovira, 1956; Timonin, 1941).

The metabolic activity of the nonrhizosphere soil microflora was stimulated by a slurry of Douglas-fir nonmycorrhizal roots (Fig. 1). After 12 hours, oxygen uptake was 1-1/2 times as great as nonrhizosphere soil plus glucose. Combining the root slurry with glucose caused a substantial increase in microbial activity as shown by increased oxygen uptake, the increase being approximately 2-1/2 times greater than the oxidation rate of nonrhizosphere soil plus glucose.

**TABLE 2. Most probable numbers of ammonifying and nitrate-reducing microorganisms**

<table>
<thead>
<tr>
<th>Rhizosphere soil type</th>
<th>Ammonifiers</th>
<th>Nitrate reducers</th>
</tr>
</thead>
<tbody>
<tr>
<td>Douglas-fir mycorrhizal root</td>
<td>74.3 Millions³</td>
<td>46.1</td>
</tr>
<tr>
<td>Douglas-fir suberized root</td>
<td>64.3</td>
<td>50.1</td>
</tr>
<tr>
<td>Red alder mycorrhizal root</td>
<td>128.8</td>
<td>47.3</td>
</tr>
<tr>
<td>Red alder suberized root</td>
<td>39.1</td>
<td>120.4</td>
</tr>
<tr>
<td>Douglas-fir nonrhizosphere soil</td>
<td>18.3</td>
<td>8.6</td>
</tr>
<tr>
<td>Red alder nonrhizosphere soil</td>
<td>17.5</td>
<td>25.3</td>
</tr>
</tbody>
</table>

1. Producing NH₄⁺ in peptone water.
2. Reducing NO₃⁻ in nitrate broth.
3. Numbers per gram of soil, oven-dry basis. Each value is the mean of the most probable number values for three replicates.
DF = DOUGLAS-FIR
MR = MYCORRHIZAL ROOT
G = GLUCOSE
NR = NONRHIZOSPHERE SOIL

Figure 1. Microbial oxidation of Douglas-fir nonmycorrhizal suberized root slurry with and without glucose (microliters of oxygen uptake beyond endogenous respiration of soil only.

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 et al., 1964) (Fig. 2). The oxidation rate was appreciably less than 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.

Metabolic activity was greatly stimulated by adding red alder mycorrhizal suspension to nonrhizosphere soil with glucose (Fig. 3). Oxygen uptake increased to 3-1/2 times that of soil and glucose alone.

The influence of red alder nonmycorrhizal suberized root slurry on the oxidative respiration patterns of nonrhizosphere soil microbes is shown in Figure 4. The microbial oxidation rate was considerably less in the presence of red alder suberized root slurry in comparison to nonrhizosphere soil plus glucose.
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 \textit{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 paper-disk technique. Slurries prepared from summer collections effectively inhibited \textit{B. cereus} and \textit{B. subtilis} on glucose-salts agar but not on peptone-beef extract agar. Slurries prepared from winter collections after leaf fall were not inhibitory on either medium.

**Discussion and Conclusions**

The data suggest that the microfloras surrounding mycorrhizae of Douglas-fir and red alder are influenced quantitatively by the fungal symbiont present.

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**Figure 2.** Microbial oxidation of Douglas-fir mycorrhizal root (\textit{Cenococcum graniforme}) slurry with and without glucose (as in Fig. 1).
The distinct differences in microbial populations found in each rhizosphere as compared with nonrhizosphere soil (Table 1) can be attributed to the influence of the mycorrhizal fungus. Population differences may be due 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. Selective absorption of organic or inorganic nutrients by the mycorrhizal fungi also may influence the rhizosphere.

The data in Table 2 show the existence of different selected physiological groups which are stimulated in each microhabitat as compared with nonrhizosphere soil. These observations are similar to those of Katznelson et al. (1962) and Neal et al. (1964). Numerical differences as related to physiological activity are not great between Douglas-fir mycorrhizal and nonmycor-
rhizal rhizospheres but do differ appreciably between these rhizospheres for alder.

The greater numbers of various microbial physiological types suggest a direct influence on availability of nutrients to the tree, particularly with respect to nitrogen. For example, a more rapid breakdown of amino acids to elemental nitrogen in rhizosphere than in nonrhizosphere soil has been reported by Katznelson and Rouatt (1957). Changes in acidity near bean roots have been observed to alter ammonium assimilation (Barker et al., 1966).

The apparent influence of mycorrhizae on associated microorganisms could possibly affect the susceptibility of roots to invasion by pathogenic fungi either by selective stimulation or inhibition of microbial groups. As suggested by Zak (1964) and Neal et al. (1964), the metabolic products of these microbial groups found in the rhizosphere also may alter the environment of the root microhabitat by changing its acidity, producing antago-
nistic materials, or selectively assimilating nutrients, thus forming an effective biological barrier to root pathogens.

Root exudates and decaying root material or sloughed-off cells are generally thought to exert the greatest influence on microorganisms in the rhizosphere (Rovira, 1965). Healthy plants, and probably diseased plants, exude a wide variety of organic compounds (Rovira, 1965). However, the amounts of exudate and cellular debris available to rhizosphere microorganisms at any one time are not known.

In our experiments, the amount of stimulation depended upon the root material associated with each microhabitat investigated. Exudates from different plants as well as different strains of the same plant species exert markedly different effects upon the rhizosphere microflora (Rovira, 1965).

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 (Fig. 1), but when glucose was incorporated with Douglas-fir mycorrhizal material, 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 in most cases is utilized preferentially by microbial cells. Thus, one could expect the oxidation rates with mycorrhizae plus glucose to be nearly the same as those obtained with soil only plus glucose or to be considerably higher as with Douglas-fir suberized roots (Fig. 1). This not being the case, however, a selective inhibition of microbial oxidation was indicated, presumably due to the antibiotic produced by the fungal symbiont, C. graniforme (Krywolap and Casida, 1964; Krywolap et al., 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 was not water soluble, the results indicate it had an active influence in the rhizosphere (Fig. 2, Tables 1, 2). The mycorrhizal fungus directly influences the rhizosphere microflora by its metabolic products and thus selectively stimulates or inhibits specific microbial groups.

The red alder mycorrhizal root slurry stimulated considerably the metabolic activity of nonrhizosphere microbes (Fig. 3). Incorporating glucose brought about an even greater response. The results indicate the mycorrhizal root material to be highly stimulating and agree 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 (Fig. 4). However, 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. Polyhydroxy phenols, reported to retard glucose oxida-
tion of selected pure cultures of bacteria (Basaraba, 1966), could possibly have been responsible for the inhibition.

A biological assay for antagonistic materials in red alder spring and summer roots and nodules (Fig. 5) revealed a substance which effectively inhibited the growth of *B. 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 toward 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

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**Figure 5. Growth inhibition of Bacillus cereus and B. subtilis by red alder root and nodule extracts on glucose-salts medium (as in Fig. 1).**
bacilli observed on glucose-salts medium. Li et al. (1968) 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 (Fig. 4) 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 (Tables 1 and 2).

Secondly, an antibiotic produced by the red alder nodule endophyte may have been present in the root and nodule extracts and effectively inhibited the growth of bacilli (Fig. 5). Based upon studies of other species of *Alnus* (Uemura, 1952a; Uemura, 1952b; and Mikola, 1965), the nitrogen-fixing endophyte of red alder nodules was presumably a *Streptomyces* species. Mikola (1965) found that several *Streptomyces* species isolated from *Alnus glutinosa* (L.) Gaertn. nodules produced antibiotics which effectively inhibited growth of *Fomes annosus* (Fr.) Cooke, 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 *A. glutinosa* endophyte 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 sublethal 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 et al. (1957) have reported that *in vitro* streptomycin is ineffective against *Phytophthora*, but when absorbed through the roots, the antibiotic confers resistance and enhances polyphenol oxidase activity. If this is the case, lack of either antibiotic production or polyphenol oxidase synthesis may be the reason no inhibitory substances were found in extracts of root and nodule material during the winter months.

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


Mikola, P. 1965. The value of adding nitrogen to forest soils. In Annual report of research conducted under grant authorized by Public Law 480. (Dep. Silvicult., Univ. Helsinki, Finland.)


