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Interaction Between Protozoa and *Rhizobium* in Chemically Amended Soil

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Interaction Between Protozoa and *Rhizobium* in Chemically Amended Soil¹

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ABSTRACT

The population of a strain of *Rhizobium phaseoli* resistant to streptomycin, erythromycin, cycloheximide, and thiram fell markedly after its addition to soil, and the numbers of indigenous protozoa rose. In sterile soil inoculated with a protozoa-free mixture of soil microorganisms, the decline in *R. phaseoli* was not as marked and was not affected by the presence of thiram. When added to nonsterile soil amended with thiram or cycloheximide, the *R. phaseoli* strain survived in larger numbers than in soil not receiving the chemicals, and the abundance of protozoa rose after an initial decline. Only one morphological type of protozoa was found in the cycloheximide-treated soil. It is suggested that the number of protozoa as well as which protozoa are active determine the population of surviving rhizobia.

Additional Index Words: predation, thiram, *Rhizobium* survival.

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A NUMBER OF BIOLOGICAL and abiotic factors affect the survival of rhizobia in soil. The possibly important biotic factors include predation (6), parasitism (9), amensalism (17), and competition (11), and the abiotic factors include pH (10), temperature (5), moisture (13, 14), and Al toxicity (10). In addition, the widespread use of fungicides to treat seeds for the control of soilborne or seedborne pathogens places another stress on the root-nodule bacteria, because although the fungicides are designed to control patho-

genic fungi, they usually are toxic to other members of the soil microflora, including *Rhizobium* (1).

The results of several investigations have suggested that predation by soil protozoa may be a major factor in the rapid decrease in numbers of rhizobia introduced into soil (6, 8). The present study was designed to obtain further evidence of the cause of the rhizobium decline after their introduction into soil and to assess the impact of antimicrobial agents on protozoan activity and the survival of *Rhizobium*.

MATERIALS AND METHODS

Rhizobium phaseoli 127K17, the test organism, is resistant to 1.0 mg of streptomycin sulfate, 50 μ g of erythromycin, 500 μ g of cycloheximide, 500 μ g of sodium dodecyl sulfate, and 500 μ g of thiram [bis(dimethylthiocarbamoyl)disulfide] ml⁻¹ (15). The bacteria were grown in 500 ml Erlenmeyer flasks containing 100 ml of yeast extract mannitol (YEM) broth (18). The bacteria were grown at 30°C for 2 days on a rotary shaker operating at 120 rpm. The cells were collected by centrifugation at 4°C and washed twice with sterile distilled water.

Lama-Honeoye silt loam (pH 7.3, 4.8% organic matter) from Amora, N.Y., was collected from the top 15 cm, allowed to dry in air, and passed through a 2 mm sieve before use. In studies of survival, 1.0 ml of a washed cell suspension was added to 10 g of air-dried soil contained in 125 ml milk dilution bottles. The number of surviving rhizobia was determined by the plate count method on YEM agar supplemented with 1.0 mg of streptomycin sulfate, 250 μ g of cycloheximide, 50 μ g of erythromycin, and 75 μ g thiram ml⁻¹. The antibiotics and thiram were sterilized by filtration.

Protozoa were counted by a modification of Singh's method (16). A petri dish was filled with 15 ml of molten 1% water agar, and five glass rings, each 20 mm in diam and 10 mm in depth, were inserted in the agar. The dishes with the rings were then sterilized by autoclaving. After the agar solidified, a *R. phaseoli* 127K17 suspension containing approximately 2×10^7 cells and 0.1 ml of a soil dilution were added to each of five rings. The plates were incubated upright for 3 days at 30°C in a moist chamber. The presence or absence of protozoa in each ring was noted by examination under low power (40 \times) magnification, and the numbers were determined by using a most probable number (MPN) table (2) with five rings cul-

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tion as the basis for the MPN count. The total number of bacteria was counted by the pour plate method, with nutrient agar as the medium. Three replicate soil samples were counted in each test, and triplicate plates were prepared for each dilution. The standard deviation in these counts was usually less than 20% of the mean.

To determine the survival of *R. phaseoli* in a protozoa-free soil, 50 g of air-dried soil was moistened with 20 ml of distilled water and incubated at 22 to 25°C. After 3 days, 200 ml of distilled water was added to the soil, and the suspension was mixed for 30 min on a rotary shaker operating at 120 rpm. The soil suspension was then filtered through Whatman no. 3 filter paper, and the filtrate was passed through a 5.0- μ m sterilized polycarbonate membrane (17 mm diam, Nucleopore Corp., Pleasanton, Calif.). The resulting filtrate was passed through a 3.0- μ m and finally a 1.0- μ m pore filter. Portions (10.0 g) of soil in 120 ml dilution bottles were sterilized by autoclaving for 2-hour periods on each of 3 consecutive days, and then the sterile soil samples were inoculated with 2.0 ml of the protozoa-free soil suspension. The sterility of the soil was shown by plating samples on nutrient agar, and the absence of protozoa in the protozoan free soil was verified by the modified Singh's method. The bottles were incubated at 30°C for 7 days, and then 1.0 ml portions of a washed cell suspension of *R. phaseoli* were added to each bottle. Plate counts were made at regular intervals of both *R. phaseoli* and total bacteria using antibiotic- and thiram-supplemented agar and nutrient agar, respectively.

In studies of the effect of thiram on the survival of *R. phaseoli* in natural or protozoa free soil, glass beads (3 mm diam, Cataphote Corp., Jackson, Mich.) were mixed with 20% (wt/vol) gum arabic. The gum was allowed to dry for about 10 min until the beads were sticky, and then thiram was mixed with the coated beads at an application rate of 0.2 g/100 g of beads. Portions (10.0 g) of soil were weighed into 120 ml dilution bottles, and one thiram-treated glass bead, 1.0 ml of *R. phaseoli* suspension containing approximately 10^6 cells, and 2.0 ml of sterile distilled water were added to each bottle. The bead was placed in the middle of the soil. Another set of soil samples received the same amendments but no thiram-coated bead.

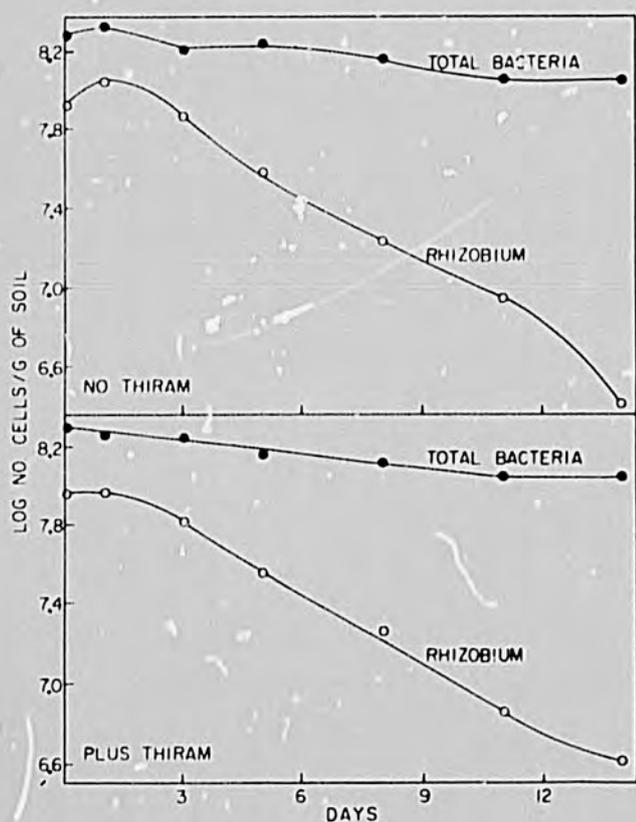


Fig. 1—Survival of *R. phaseoli* 127K17 in sterile soil inoculated with a protozoa-free mixture of soil microorganisms and *R. phaseoli* in the absence and presence of thiram.

In an experiment in which cycloheximide was used, it was mixed with the soil to a final concentration of 0.5 mg/g of air-dried soil. Each 120 ml milk dilution bottle contained 10.0 g of soil, and each was inoculated with 1.0 ml of a *R. phaseoli* suspension containing approximately 5×10^6 cells and 2.0 ml of sterilized distilled water.

Unless otherwise stated, the bottles were incubated at 30°C in the dark.

RESULTS

When *R. phaseoli* was introduced into soil, its population declined and then reached a reasonably constant level; for example, in a soil receiving about 10^6 cells/g, the number reached about 10^4 g at 4 days and did not change greatly thereafter. At the time of the decline, the protozoan numbers rose to about 10^4 g of soil. These data are in agreement with previous findings (6, 7), except for the somewhat lower numbers of surviving bacteria.

To assess the impact of protozoa, a study was conducted of rhizobial survival in a sterile soil that was inoculated with a protozoa-free mixture of microorganisms. Approximately 10^6 cells of *R. phaseoli* were added to 10 g of this protozoa-free soil. The number of *R. phaseoli* decreased from about 9×10^7 to 5×10^6 cells/g of soil in a 14-day period (Fig. 1, top). Such data are in marked contrast with the appreciable decline in rhizobial abundance in normal soil; e.g., *R. phaseoli* declined from 5.2×10^7 to 7.3×10^5 cells/g of soil in only 3 days (Fig. 2, top).

It has been reported that legumes inoculated with fungicide-resistant rhizobia give greater yields if the

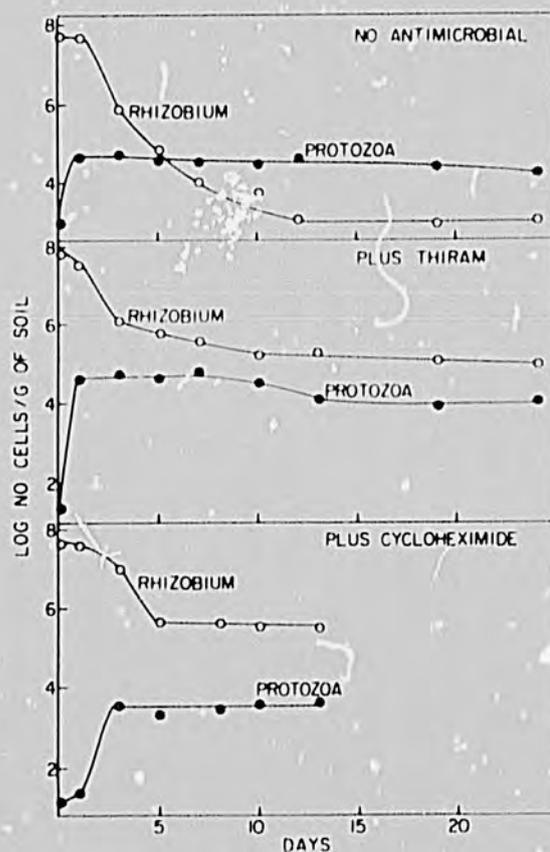


Fig. 2—Survival of *R. phaseoli* 127K17 and protozoan numbers in nonsterile soil that did not receive antimicrobial chemical, thiram, or cycloheximide.

seeds are treated with the fungicides than if they are not so treated (12). If protozoa are important in the decline of the rhizobia, it is possible the fungicide, which often acts on a variety of microorganisms in addition to fungi (1), may inhibit the protozoa and thus favor colonization by the rhizobia. This possibility was tested by adding a *R. phaseoli* cell suspension to protozoa-free soil with thiram treatment. It is evident from the results in Fig. 1 that the population decline was essentially the same whether or not the soil received the fungicide.

The changes in *R. phaseoli* numbers were quite different if protozoa were present. Thus, it is evident from the results in Fig. 2 (center) that the numbers of surviving rhizobia were markedly affected by the fungicide. At the end of 21 days, about 1×10^5 cells/g of soil were present if the soil received thiram, but only 1×10^3 cells/g of soil were present if the soil received no fungicide. Moreover, although the initial number of protozoa in the thiram-treated soil was much lower than in the untreated soil, a difference probably attributable to the killing of sensitive protozoa by thiram during the 2-hour period that elapsed as all the bottles received the amendments, the protozoan counts in both soil samples reached a level of about 6×10^4 cells/g of soil, and the final counts were also similar at about 2×10^3 cells/g of soil.

To determine whether the great difference in abundance of survivors between the unamended and thiram-amended soil containing protozoa was in fact an effect of thiram on protozoan activities, a similar experiment was performed using cycloheximide (actidione) in place of the fungicide. This antibiotic was chosen because it is toxic to protozoa, although it also affects other eukaryotic organisms. The results are given at the bottom of Fig. 2. At day 1, when the number of protozoa was still low, the *R. phaseoli* population did not appreciably decline from that initially added. However, when the number of protozoa rose to about 4×10^3 cells/g of soil, the abundance of *R. phaseoli* was noted to have fallen. Microscopic examination of the cycloheximide-treated soil revealed that the diversity of protozoa had declined abruptly, and only one morphological type was detected, a ciliate.

DISCUSSION

The behavior of *R. phaseoli* in protozoa-free and natural soil was quite different. In the former instance, the numbers did not decline abruptly. In the natural soil, in contrast, a dramatic fall in abundance was evident, and the decline was correlated with the rise in numbers of protozoa. These results support the view that protozoa are responsible for the rapid decline of a *Rhizobium* population shortly after it is introduced into soil. The finding that the *Rhizobium* population did not decline in the first day in natural soil as the protozoan number increased may be attributable to the burst of bacterial growth when the soil was moistened; in this initial period, the protozoa probably are feeding on the large numbers of indigenous bacteria. However, as the size of the indigenous bacterial community was reduced by predation, the preying on the rhizobia may have become pronounced.

A 100-fold difference in the final rhizobial numbers between thiram-treated and untreated soil was ob-

served. However, in protozoa-free soil, whether it was treated with thiram or not, the curves of survival of *R. phaseoli* 127K17 were the same.

Protozoa consume large quantities of bacteria per cell division (3), but the final number of surviving prey varies with the protozoan species (6, 8). It is evident from this study that certain chemicals reduced protozoan numbers immediately after they were added. Shortly thereafter, however, no appreciable difference was evident between the number in the chemically treated and untreated soil; nevertheless, the protozoan communities may be quite dissimilar as a result of addition of the chemical. Hence, different numbers of rhizobia probably will survive according to how the toxic chemical alters the composition of the protozoan community. Nevertheless, further research is required to establish whether changes in protozoan fauna affect the density of surviving rhizobia and whether these findings have practical utility in promoting nodulation.

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