Interaction of Rhizobium sp. with Toxin-Producing Fungus in Culture Medium and in a Tropical Soil†

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Experiments were conducted to determine the influence of a toxin-producing fungus on a rhizobial population in yeast-mannitol medium and in a tropical soil. The fungus, which was isolated from a highly weathered soil (Tropeptic Eutrustox), was identified as a Metarhizium sp. The density of rhizobial populations established in yeast-mannitol medium in the absence of the fungus was 10^5 times higher than that established in its presence. However, the fungus did not exert similar antagonistic influence on the rhizobial population incubated with it in the sterilized test soil. Rhizobial growth activity in yeast-mannitol medium was also insensitive to the presence of the fungus when the medium was amended with 1% (wt/vol) kaolinite or montmorillonite. The results suggest that clay minerals may be responsible for protecting rhizobia against toxin-producing fungi in soil.

The populations of useful microorganisms introduced into the soil may be reduced in size by activities of soil microorganisms. One of the mechanisms by which the indigenous population may bring about such a reduction involves the production of toxic metabolites.

Toxin-producing fungi that affect rhizobial populations are ubiquitous in soil and often are readily isolated on laboratory media (3, 10, 11, 13). In some instances, the occurrence of these fungi in soil or their deliberate addition to soil has been reported to result in decreased nodulation and nitrogen fixation in legumes (2, 3, 7). Recently, Angle et al. (1) have reported that the presence of mycotoxin-producing fungi in soil was responsible for declines in Rhizobium japonicum populations as well as decreases in nodulation, N₂ fixation, and growth of soybean.

The extent to which toxin-producing fungi influence Rhizobium sp. and its symbiotic properties is variable, depending on the Rhizobium species and strains, the types of host legumes, and the types of fungi involved (1, 3, 6, 11). This paper describes the results of experiments designed to determine the influence of a toxin-producing fungal isolate from a highly weathered tropical soil on a Rhizobium strain in culture medium and in a tropical soil.

MATERIALS AND METHODS

Soil. The soil used in this study was a Wahiawa silty clay loam (Tropeptic Eutrustox). It was collected from the Pamo-ho Experiment Farm, College of Tropical Agriculture, Island of Oahu, Hawaii. Surface soil samples (0 to 10 cm) of the soil were crushed to pass through a 2.0-mm sieve. The pH of the soil was 5.9. Sterile soil was obtained by exposing 10-g portions of the soil contained in screw-capped test tubes to 2.5 Mrads of γ radiation from a cobalt-60 source.

Chemicals. Cycloheximide, griseofulvin, nystatin, and streptomycin were purchased from Sigma Chemical Co., St. Louis, Mo. Chlorneben was supplied by E. I. duPont de Nemours & Co., Wilmington, Del.

Media. Yeast-mannitol medium (14) was used for maintaining, culturing, and enumerating rhizobia and for interacting rhizobia with the toxin-producing fungus. Malt extract agar (8) was used for cultivating and maintaining the toxin-producing fungus.

A saline solution containing 0.5 g of K₂HPO₄, 0.2 g of MgSO₄·7H₂O, and 0.1 g of NaCl per liter of distilled water was used for washing and suspending rhizobial cells and for suspending fungal spores.

Cultures. Rhizobium sp. (Tal 582) was obtained from the culture collection of the NiTAL Project, University of Hawaii. The organism is a strain specific to leucaena (Leucaena leucocephala). Stock cultures were maintained on yeast-mannitol agar (YMA) slants at 4°C. A spontaneous mutant of the culture that was resistant to 500 μg of streptomycin was obtained as described by Habte and Alexander (6).

Rhizobial cell suspensions for the various experiments were obtained from 6-day-old cultures grown on YMA slants or in yeast-mannitol broth (YMB) at 30°C. Cultures from slants were directly suspended in sterile saline solution, and those from liquid medium were harvested by centrifugation at 8,000 × g and washed twice in saline solution, and the pellet was brought back to original volume with saline solution.

The toxin-producing fungus attracted our attention when its frequent occurrence on YMA plates amended with 200 μg of cycloheximide, 50 μg of chloronene, and 500 μg of streptomycin per ml led to poor recovery of rhizobia, apparently owing to the suppression of the bacteria by the fungus. Subsequent confirmation of toxin production by the fungus and its isolation in pure culture was made as follows. Soil dilutions of the Wahiawa soil were amended with a washed suspension of Rhizobium sp. Tal 582SR to obtain about 3,000 colonies of the test bacterium per plate. One-millimeter portions of the soil suspension were then inoculated into sterile petri plates into which melted YMA containing 500 μg of streptomycin and 200 μg of cycloheximide per ml was poured. The plates were incubated at 30°C. Individual colonies of the fungus exhibiting zones of rhizobial inhibition were picked up and further purified by streaking on YMA, and then single colonies were transferred onto malt extract agar slants. The slants were incubated at 30°C until their

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surfaces were covered with spores. Slants were then stored at 4°C until needed. The fungus was identified as Metarhizium sp. by using the diagnostic features outlined by Barron (2) and Domsch et al. (5). Spore inocula were prepared by aseptically scraping off the spores from the malt extract agar slant surface into saline solution and then diluting the suspension to obtain a total volume of 40 ml. This suspension was further diluted as needed to yield appropriate initial spore concentrations for the various experiments.

Interaction in culture medium. For determination of the influence of the toxin-producing fungus on rhizobia in culture medium, 1.0-ml portions of the fungal spore suspension containing $6.7 \times 10^4$ spores per ml was mixed with 1.0-ml portions of rhizobial suspension in 100 ml of quarter-strength YMB (pH 6.2) contained in 250-ml Erlenmeyer flasks. The medium contained all the ingredients specified for YMB except that it was supplemented with 0.05% (wt/vol) griseofulvin and nystatin. The rhizobial suspension was obtained by aseptically plating one 10-ml portion of the bacteria into 100 ml of YMA containing 50 µg each of griseofulvin and nystatin per ml of medium. CFU of the toxin-producing fungus were obtained by plating the suspension on YMA and incubating for 2 to 12 days before colonies were counted.

Interaction in soil. For studying the influence of the toxin-producing fungus on Rhizobium sp. in soil, 1.0-ml portions of the suspension of the bacteria with or without 1.0-ml portions of the suspension of the fungal spores containing $2.5 \times 10^4$ to $5.3 \times 10^4$ spores per ml were inoculated into 10-g portions of the y-irradiated Wahiawa soil contained in 160-ml foam-plugged dilution bottles. The moisture content of the soils was adjusted to 60% of water holding capacity. The bottles were placed in zip-lock bags containing a thin layer of water, and the bags were incubated at 30°C. At regular intervals, three of the bottles from each treatment were withdrawn. Serial dilutions of the samples were made, and the numbers of the interacting populations were determined as described above.

FIG. 1. Influence of toxin-producing fungus on Rhizobium sp. Tal 582SR in culture medium. LSD, Least significant difference.

FIG. 2. Interaction of Rhizobium sp. Tal 582SR at low (a) and high (b) initial densities with toxin-producing fungus in sterile Wahiawa soil. LSD, Least significant difference.
RESULTS AND DISCUSSION

When Rhizobium sp. Tal 582SR was incubated with the toxin-producing fungus in quarter-strength YMB, the results shown in Fig. 1 were obtained. In the absence of the fungus, Tal 582SR cells multiplied rapidly and reached a maximum number of about 10^8 cells per ml in 6 days. This population level was maintained until the end of the experiment. In the presence of the fungus, the density of Tal 582SR exhibited a 40-fold increase during day 1 of incubation. This increase was followed by a decline which brought the population back to its initial level. The population level of Tal 582SR maintained in the absence of the fungus was 10^7 times higher than that maintained in the presence of the fungus. These results demonstrate that the rhizobial population was significantly suppressed by the fungus in culture medium. Although the population was drastically suppressed by the fungus, the ability of the bacterium to maintain itself at low population levels in the presence of its antagonist is interesting. These low numbers of rhizobial cells coexisting with their antagonist, however, did not appear to be inherently resistant to the toxic action of the fungus, since they were not found to be insensitive to the presence of the fungus upon concentration and subsequent exposure to it. Because the fungus tended to form clumps in YMB, counts of its CFU tended to be irregular and hence are not reported here.

Figure 2 shows the results obtained when the Rhizobium population was interacted with the toxin-producing fungus in sterile soil. The population of Tal 582SR grew in the Wahiawa soil and stabilized itself at a level which is normal for rhizobia in sterile soil (4, 6) irrespective of the presence of the fungus and regardless of the initial density of Rhizobium sp. used. Growth and survival of Tal 582SR in the sterile Wahiawa soil, therefore, was not influenced by the toxin-producing fungus. The fungus was also able to grow in the sterile soil and stabilized its population at about 5 x 10^7 cells per g, a level comparable to climax values reported for many soil fungi. Comparable results were obtained in similar studies in which Rhizobium sp. Tal 2095SR (from the cowpea miscellany group) was used in the Wahiawa soil or when both Tal 2095SR and Tal 582SR were used in a tropical Haplustoll (Habte and Barrion, unpublished data).

The remarkable contrast between the ability of the toxin-producing fungus to effectively suppress the test Rhizobium sp. in culture medium and its inability to exert a similar influence in sterile soil suggests that soil constituents may be protecting the rhizobial cells against the antagonistic influence of the fungus. An experiment was therefore conducted to test the possible influence that clay minerals might have in protecting rhizobia against the toxin-producing fungus (Fig. 3). The results show that the fungus, as expected, suppressed the population of Tal 582SR significantly in the absence of the clay minerals, whereas in the presence of either kaolinite or montmorillonite, the bacterium was able to maintain a population level comparable to that which it had attained previously in quarter-strength YMB in the absence of the fungus (see Fig. 1). These results clearly demonstrate that clay minerals can play important roles in protecting rhizobia against toxin-producing fungi in soil. The similarity exhibited by the two clay minerals in protecting rhizobia, despite the much higher specific surface and negative charge of montmorillonite (9), is unexpected. It is possible, however, that the quantity of toxic material produced did not exceed the amount that could be adsorbed by the quantity of kaolinite used in this experiment. This observation is supported by the fact that kaolinite was found to be significantly inferior than montmorillonite in protecting Tal 582SR from the toxin-producing fungus in YMB where each clay mineral was added to the medium at a concentration four times lower than that employed in the above experiment (data not shown).

Our evidence supports the earlier findings of Skinner (12), who found that the antagonistic influence of Streptomyces albidoflavus is decreased when bentonite is added to sand culture or when the two populations are interacted in sterile soils. Our findings, however, are in disagreement with those of Angle et al. (1), in which significant reductions in R. japonicum populations were attributed to inoculation of soil with antagonistic fungi. Differences in the rhizobial species employed and the types of antagonistic fungi involved, as well as the nature of the toxic chemicals elaborated by the different fungi, could lead to dissimilarities in the results observed by different investigators. However, antagonistic fungi producing toxic compounds similar to those that can be produced by the fungal isolate we tested here are not expected to influence rhizobial survival and function in mineral soils containing >1% clay.

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LITERATURE CITED