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Method for Establishing a Bacterial Inoculum on Corn Roots

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Few bacteria from the corn rhizosphere grew in media with 50 μg of mancozeb per ml. A mancozeb-resistant *Pseudomonas* strain from the rhizosphere was serially subcultured in media containing mancozeb and spectinomycin until it was resistant to 175 μg of mancozeb and 850 μg of spectinomycin per ml. The population of the pseudomonad added to soil fell to low numbers in 6 days in unamended or glucose-amended soil, but its numbers exceeded 10^5/g for at least 12 days if the soil was supplemented with mancozeb. The numbers of this organism remained small on corn roots derived from untreated, inoculated seeds, but the population was two or more orders of magnitude greater on roots derived from mancozeb-coated seeds. The abundance of the inoculum strain on the 3-cm portion of roots nearest the stem declined markedly after about 1 week, but applying urea to the foliage reduced or prevented the decline. The numbers of the pseudomonad on segments of roots 3- to 6- and 6- to 9-cm from the stem were higher on plants derived from the mancozeb-coated seeds. Applying spectinomycin to the foliage did not promote growth of the bacterium. This method is proposed as a means to establish an introduced bacterium on plant roots.

Microorganisms are beneficial to plant growth in many ways. By mineralizing organic forms of nitrogen, phosphorus, and sulfur that roots cannot use as nutrients, the rhizosphere microflora provides inorganic ions that sustain plant growth. The rhizosphere inhabitants, in culture at least but possibly also in nature, are known to synthesize plant growth regulators (8) and products toxic to soil-borne plant pathogens (24). Many studies have demonstrated that bacteria associated with the roots of nonlegumes may also bring about N_2 fixation (3).

Because of the benefits that the rhizosphere inhabitants can provide, many attempts have been made to enhance the processes that these microorganisms effect. One approach to bring about such an enhancement is to inoculate plants with a species active in the particular transformation. Particular attention has been given to nitrogen fixation; thus, *Azotobacter chroococcum* has been inoculated onto wheat seeds (18), *Azospirillum* sp. has been added to millet (1), and *Beijerinckia derxii* has been applied to wheat (19). Attempts have also been made to increase phosphorus uptake of plants by use of inocula that contain bacteria which solubilize insoluble phosphates (13). Strains of *Pseudomonas* (6), *Bacillus*, and *Streptomyces* (15) have also been applied to seeds of cereal or vegetable crops to enhance plant growth. In most instances, no means was employed to selectively favor growth of the bacterium used as inoculum, and it was assumed that the microorganism would be able to establish itself on the roots in competition with the indigenous microflora solely because it was added to the seed in large numbers. It has recently been shown, however, that the growth or survival of *Rhizobium phaseoli* in the rhizosphere of beans may be enhanced by applying a fungicide and a fungicide-resistant strain of *R. phaseoli* to seeds (14, 16). The fungicide, in addition to suppressing many bacteria that would compete with the inoculum strain for plant exudates, markedly reduces the population of protozoa that might prey on the rhizosphere colonizers. As a result, nitrogen fixation and bean growth were increased.

The present study was designed to determine whether the application of a fungicide and a fungicide-resistant bacterium would lead to enhanced colonization of the roots of a cereal. In contrast with *Rhizobium* spp., the bacterium in this instance does not enter into the root and thus must survive prolonged interactions with other microorganisms growing on root surfaces.

MATERIALS AND METHODS

Obtaining resistant isolates. Dilutions of soil from the rhizosphere of corn grown in Lima silt loam were plated on nutrient agar containing mancozeb (Mn and Zn salt of ethylene bis[dithiocarbamate]; 91.6% pure;

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E. I. DuPont de Nemours & Co., Inc., Wilmington, Del.), thiram (tetramethylthiuram disulfide; 97% pure; Aldrich Chemical Co., Milwaukee, Wis.), spectinomycin (GIBCO Laboratories, Grand Island, N.Y.), or streptomycin (Nutritional Biochemicals Corp., Cleveland, Ohio) in a range of concentrations. The plates were incubated for 48 h at 28°C, and then bacteria growing rapidly on plates which had the highest chemical concentrations and few colonies were transferred to nutrient agar containing one of the antimicrobial agents. To obtain isolates of greater resistance, the bacteria were grown in nutrient broth for 48 h at 30°C on a rotary shaker, and the cells were collected by centrifugation and washed three times with 0.1 M phosphate buffer (pH 7.0). An inoculum containing 3.8×10^9 to about 1×10^{10} of these washed cells per ml was added to 100-ml Erlenmeyer flasks containing 50 ml of nutrient broth supplemented with, in subsequent transfers, mancozeb, streptomycin, thiram, and spectinomycin in increments increasing by 25, 50, 100, and 100 μg, respectively.

The pseudomonad that was thus isolated was routinely grown in a medium (SMS) containing the following (per liter of distilled water): sucrose, 10 g; yeast extract, 0.5 g; Casamino Acids, 0.5 g; K_2HPO_4, 0.6 g; $(NH_4)_2HPO_4$, 0.4 g; $MgSO_4 \cdot 7H_2O$, 0.2 g; $CaCl_2$, 20 mg; $FeCl_3$, 2 mg; and agar, 15 g. The pH after autoclaving was 7.0. When used, sugars, antibiotics, and fungicides were sterilized by filtration through 0.22-μm GS filters (Millipore Corp., Bedford, Mass.) and then added to the agar media. Before sterilization, streptomycin and spectinomycin sulfates were dissolved in demineralized water, mancozeb was dissolved in 1% Na-EDTA solution, and thiram was dissolved in chloroform.

The bacteria were counted on nutrient agar or SMS medium by the pour-plate method, the plates being incubated for 72 h at 28°C. The medium in which the pseudomonad in soil or rhizosphere was counted contained the specific chemicals at the highest levels to which the bacterium was resistant, which were 175 μg of mancozeb and 850 μg of spectinomycin per ml.

Effect of glucose and mancozeb amendment. Triplicate 25-g portions of Lima silt loam (pH 7.1) from Aurora, N.Y., were placed in 250-ml Erlenmeyer flasks. The soil was from the top 15 cm and was passed through a 0.2-mm sieve before use. The soil samples were inoculated with 3×10^4 pseudomonads per g and amended with (i) nothing, (ii) 1% glucose, (iii) 175 μg of mancozeb per g, or (iv) glucose and mancozeb. The soils were incubated at 28°C for 12 days at 35% of their water-holding capacity, and counts were made every 2 days, using all the soil in each flask to prepare the initial dilution. The counts were made on SMS agar and SMS agar containing 175 μg of mancozeb per ml.

Root colonization. Pseudomonad cells from 5.0 ml of a 48-h liquid culture were collected by centrifugation, washed three times with 0.1 M phosphate buffer (pH 7.0), and suspended in 50 ml of a 40% aqueous gum arabic solution. This solution was mixed for 2 min with 200 corn seeds (Northrup King variety PX20, pesticide free), and the seeds were then allowed to dry for 1 h. Each seed was inoculated with 7×10^4 cells, and half of the seeds were treated with mancozeb (about 2 mg per seed). Seven replicate groups, each with 20 seeds of similar size, were then placed in sterile plastic petri dishes and covered with 100 g of Lima silt loam, and the dishes were incubated for 12 days in a Biotronette Mark III environmental chamber (Lab Line Instruments, Melrose Park, Ill.) under 12 h of light providing 3.0 μEinsteins/s per m^2 at 28°C and 12 h of darkness at 21°C. The soil moisture was kept at about 35% of the water-holding capacity.

Groups of 10 seedlings chosen at regular intervals were gently removed from the soil with forceps, and the seedlings were shaken to detach soil not strongly adhering to the roots. The roots were then separated from the seeds with scissors, and dilutions and counts were made separately for the seeds and for the entire roots. Dilutions prepared for every two seeds or seedlings were plated on SMS agar containing 175 μg of mancozeb per ml, and counts of the pseudomonad were made on plates incubated at 28°C for 4 days.

Movement of pseudomonad with roots. Four replicate groups of seeds were inoculated with 700 cells per seed, treated with about 1.2 mg of mancozeb per seed, and sown in plastic pots (11.5 cm diameter by 7 cm high, one seed per pot) containing 600 g of soil. The plants were grown for 26 days in the environmental chambers, and counts of five plants were made at regular intervals. To perform the counts, the plants were carefully removed from the pots with sterile forceps and gently shaken to dislodge soil not strongly attached to the roots, and then the roots were cut into 3-cm sections. The root pieces were placed in tubes containing 10 ml of sterile distilled water, with no more than 5 pieces of the same root section in a tube. The samples were mixed for 5 min at about 420 cycles per min with a Lab Line super mixer (Arthur Thomas, Philadelphia, Pa.), and dilutions were prepared and plated on SMS agar containing 175 μg of mancozeb per ml. The dishes were incubated at 28°C for 4 days before counting.

On day 8 and at 4-day intervals thereafter, the foliage of groups of the plants was sprayed twice with a solution of 0.1% Tween 80 in water or 0.1 M urea and Tween 80. The pots were watered 3 h before each spraying. The spraying was conducted with an atomizer, and both sides of the leaves were treated. Each leaf received about 0.1 ml of liquid. To prevent drops from the sprayed leaves from falling onto the soil, we placed Whatman no. 3 filter paper disks around the stem of each plant above the soil. Root samples were prepared as described above, and the pseudomonads on the 3-cm portion of the roots nearest the stems and on the 3- to 6- and 6- to 9-cm segments were counted, using SMS medium containing mancozeb.

Effect of seed and foliar treatment on introduced and indigenous bacteria. Uninoculated and inoculated seeds treated or not treated with mancozeb were sown in soil in plastic pots, and the pots were placed at random in the environmental chambers and watered every 2 days. At 4-day intervals, the plants were watered with 20 to 40 ml of a nutrient solution containing 22.8 g of NH_4NO_3, 20.7 g of K_2HPO_4, and 19.7 g of KH_2PO_4 per liter of distilled water (23). On day 5 after germination, each leaf was sprayed with about 0.1 ml of liquid. The seedlings derived from inoculated, fungicide-treated seeds received a 0.1% Tween 80 solution or 0.1 M urea in the Tween 80 solution. The seedlings growing from inoculated seeds not treated with mancozeb were sprayed with 850 μg of spectinomycin or 1.0 mg of terrazole (5-ethoxy-3-trichloromethyl-2,2,4-thiodiazole) (Mallinckrodt Inc., St. Louis, Mo.) per ml

TABLE 1. Effect of antimicrobial chemicals at different concentrations in nutrient agar on counts of bacteria from corn rhizosphere

Chemical concn (μg/ml)	No. of bacteria per g of soil			
	Mancozeb	Thiram	Spectinomycin	Streptomycin
0	53.8[a]	30.5[a]	36.3[a]	26.4[a]
50	28	ND[b]	195	ND
100	3	ND	141	ND
150	0	ND	120	248
200	0	128	94	248
300	0	51	41	124
400	0	27	21	56
500	0	10	3	34
600	0	0	0	4

[a] Counts in millions of cells per gram. All other values are actual numbers.
[b] ND, Not determined.

of the Tween 80 solution. The roots were removed from the soil, and counts of the introduced and native bacterial populations on the 3-cm root portion nearest the stem were made before and after the foliage was sprayed. Each treatment was replicated four times, and bacterial counts were made of each plant.

RESULTS

Isolation of resistant bacteria. Dilutions of soil from the corn rhizosphere were plated on triplicate plates of nutrient agar to which mancozeb, thiram, spectinomycin, or streptomycin had been added. The colonies were counted after 3 days of incubation at 30°C. At any one concentration of the four antimicrobial chemicals, mancozeb permitted the growth of the fewest colonies (Table 1). Essentially the same results were obtained for counts made from samples of the alfalfa and soybean rhizospheres, mancozeb being the most effective of the chemicals against the bacteria from the rhizosphere of each plant species.

Six dissimilar colonies were selected from the plates, and these bacteria were subcultured repeatedly in nutrient broth containing increasing concentrations of the chemicals (first mancozeb and then either spectinomycin or streptomycin) above the levels to which the bacteria were already resistant. An isolate was chosen that was resistant to 175 μg of mancozeb and 850 μg of spectinomycin per ml. This bacterium was a gram-negative, nonspore-forming, straight rod, 0.5 to 0.7 μm wide by 1.0 to 1.8 μm long, that was motile and possessed 1 to 5 polar flagella. It produced a fluorescent pigment in King B medium and a yellow pigment in SMS medium. It gave positive tests for catalase, urease, peroxidase, use of nitrate as a nitrogen source, nitrite and ammonium formation from nitrate, gelatin liquefaction, and the presence of poly-β-hydroxybutyrate granules. Gas was not produced from several sugars, and N_2 was not formed from nitrate. These characteristics indicate that the bacterium is a strain of *Pseudomonas* (5, 17). It was designated *Pseudomonas* sp. strain MMR51.

Pseudomonas sp. strain MMR51 retained its viability when suspended in 0.1 M phosphate buffer (pH 7.0) at 28°C on a rotary shaker operating at 200 rpm. Thus, with a washed inoculum of 9.5×10^6 cells per ml derived from a 48-h culture grown in SMS broth, the population was 2.1×10^6, 1.1×10^6, and 0.48×10^6/ml at 7, 18, and 30 days, respectively. Hence, the bacterium is resistant to starvation.

Addition of glucose and mancozeb to soil. To determine whether this bacterium could become established if the indigenous soil community was suppressed by an antimicrobial agent to which the added organism was resistant, a suspension of *Pseudomonas* sp. strain MMR51 was introduced into soil that had been treated with glucose alone, mancozeb alone, a combination of glucose and mancozeb, or no chemicals. Dilutions of the inoculated soils were plated, in triplicate, on SMS agar with and without mancozeb. The total counts of bacteria able to grow on fungicide-free agar were initially low, the reason for which is unknown (Fig. 1). In soil receiving no mancozeb, counts of these bacteria rose slowly if no sugar was added but increased more rapidly and reached higher values if the soil was supplemented with glucose. In soil treated with mancozeb, the total number of bacteria rose somewhat but increased markedly if sugar was also added to the soil. The data also show that *Pseudomonas* sp. strain MMR51 fell to low numbers by day 6 if the soil was not amended with mancozeb, although replication initially occurred when glucose was introduced into the soil. In contrast, *Pseudomonas* sp. strain MMR51 populations were maintained at higher levels in the mancozeb-treated soil, whether or not glucose was also added.

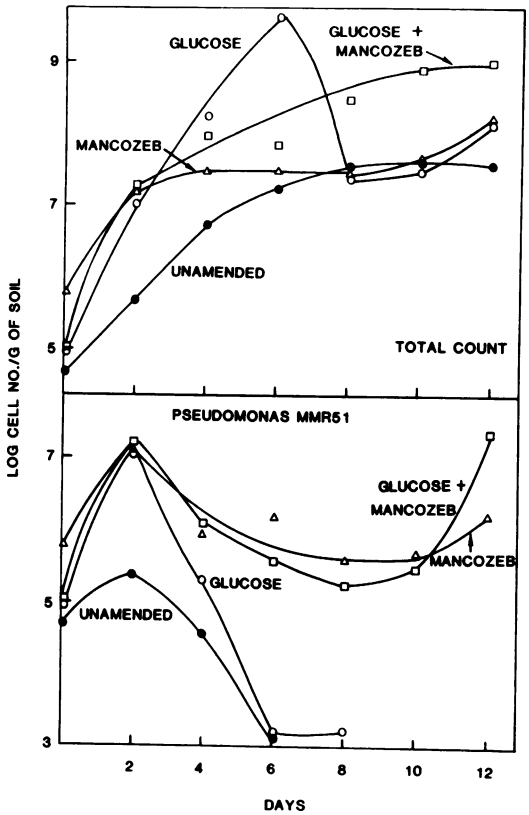

FIG. 1. Populations of total bacteria and *Pseudomonas* sp. strain MMR51 in unamended soil and in soil amended with glucose, mancozeb, or glucose plus mancozeb.

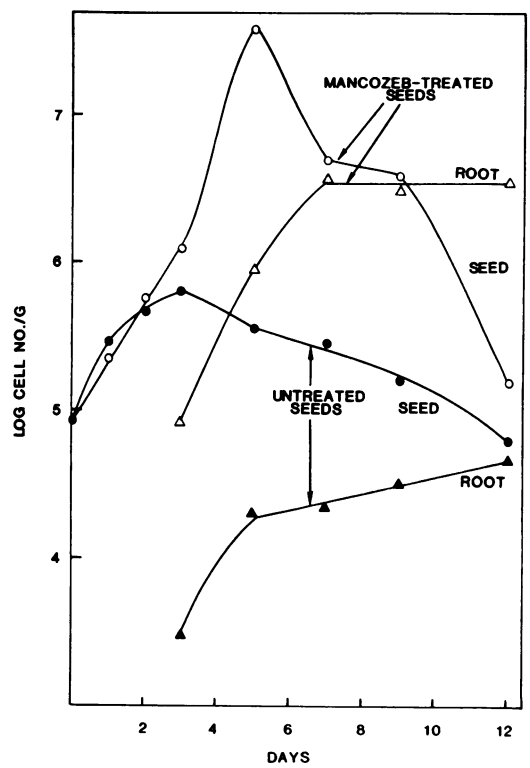

FIG. 2. Colonization of corn roots and seeds by *Pseudomonas* sp. strain MMR51 inoculated onto untreated or mancozeb-treated seeds. The counts are per gram of seed or per gram of root system.

Colonization of roots. Colonization of corn seeds and roots was measured with seeds that were inoculated with the pseudomonad and either coated with mancozeb or left uncoated. The data show that the size of the *Pseudomonas* sp. strain MMR51 population on the seeds increased during the first few days and then decreased, but the number of cells was far greater on the mancozeb-treated than on the unamended seeds (Fig. 2). Similarly, the number of cells on roots derived from mancozeb-coated seeds was far greater than on roots from uncoated seeds; indeed, the population was often two orders of magnitude larger. Thus, coating the seeds with mancozeb favored the establishment and survival of the resistant bacteria on both seeds and roots.

Movement of pseudomonad with roots. The movement of bacteria from the inoculated seed with the emerging root was determined with seeds treated or not treated with mancozeb. The foliage of some of the plants was sprayed with urea on day 8, and the spray was repeated thereafter at 4-day intervals. The population of *Pseudomonas* sp. strain MMR51 on the 3-cm portion of mancozeb-treated roots nearest the stem (designated 0 to 3 cm) increased more quickly than that on roots derived from fungicide-free seeds (Fig. 3). However, the population sizes on both treated and untreated roots decreased after days 6 and 7. If urea was applied to the foliage of plants derived from the mancozeb-coated seeds, the extent of the decline in numbers was reduced, and a larger *Pseudomonas* sp. strain MMR51 population was maintained. The pseudomonad was also found on the 3- to 6- and 6- to 9-cm portions of the roots, and here too its numbers were greater if the seeds were coated with the fungicide. The effect of urea was evident also on the 3- to 6-cm segments. The beneficial effect of urea may be related to improved plant growth because urea in separate experiments was found to cause statistically significant increases (95% confidence interval) in plant weight at harvests made on days 7, 11, 13, 17, and 21.

Effect of seed and foliar treatment on introduced and indigenous bacteria. Mancozeb-treated and untreated seeds were inoculated with

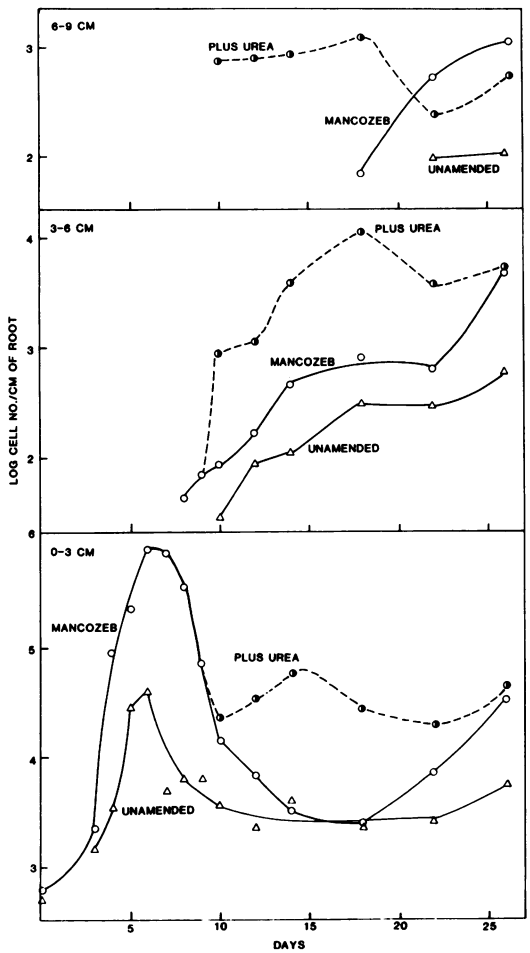

FIG. 3. *Pseudomonas* sp. strain MMR51 populations on 0- to 3-, 3- to 6-, and 6- to 9-cm portions of corn roots derived from untreated or mancozeb-treated seeds. The foliage of half of the corn plants emerging from fungicide-coated seeds was sprayed with urea.

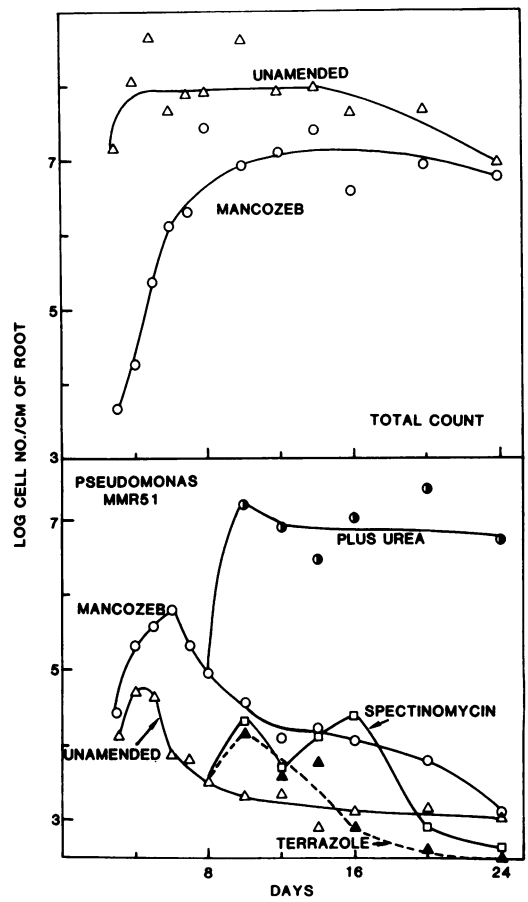

FIG. 4. Counts of total bacteria and *Pseudomonas* sp. strain MMR51 on corn roots derived from mancozeb-coated and uncoated seeds and of the pseudomonad on roots of plants having the foliage treated with urea, spectinomycin, or terrazole.

Pseudomonas sp. strain MMR51. After 5 days, the seedlings from the treated seeds were sprayed every 12 h with urea, and seedlings growing from fungicide-free seeds were sprayed with a solution containing urea and spectinomycin or a solution containing urea and terrazole. One group of seeds was not chemically treated. Each foliar spray was applied 4 times, and counts were made on the 0- to 3-cm portion of the roots.

The counts of total bacteria on the root segments were high if the seeds were not coated with the fungicide (Fig. 4). The total bacterial count on the roots derived from the mancozeb-coated seeds was initially low, but then the numbers increased but were still below those on seedlings arising from untreated seeds. The *Pseudomonas* sp. strain MMR51 population counted on SMS agar amended with mancozeb was again higher on plants derived from mancozeb-treated seeds than it was on plants from uncoated seeds, and in this instance, treatment with urea even more markedly enhanced the growth and survival of the pseudomonads. Although *Pseudomonas* sp. strain MMR51 was spectinomycin-resistant, spraying the foliage with this antibiotic resulted only in a slight and transitory increase in numbers; in this instance, the agar medium used for counting contained both mancozeb and spectinomycin. Similarly, application of terrazole, which presumably is translocated downward, had only a transitory effect.

DISCUSSION

These findings show that it is possible to selectively enhance the proliferation of specific

organisms that are resistant to a chemical that inhibits the normal inhabitants of the root zone. It is not clear from the data presented whether the pseudomonad is favored because the inhibitor suppresses bacteria competing with the pseudomonad for root and seed exudates or because the inhibitor suppresses protozoa or other predators and parasites that feed on the pseudomonad, or both. However, the marked fall in numbers of *Pseudomonas* sp. strain MMR51 after the initial period of proliferation is probably not a consequence of competition, because populations of this bacterium do not decline readily when starved. The decline is similar to that observed in the bean rhizosphere for *R. phaseoli*, a population decrease that has been ascribed to predation by protozoa (14, 16). The inability of Döbereiner and Divan Baldani (3) to obtain growth of streptomycin-resistant strains of *Azospirillum lipoferum* and other bacteria on corn roots may have resulted from their use of a cell density far too large to show replication.

Applying urea to the foliage also was beneficial to the introduced pseudomonad. Vrany et al. (22) reported that the number of *Pseudomonas putida* in the rhizosphere of wheat was increased by applying urea to the foliage. Urea may be beneficial because it alters the amount and composition of root exudates as a result of its effects on photosynthesis (7, 10). Because urea and other foliar sprays alter the quantity and composition of the root exudates as well as the microflora living on them (9, 20), such procedures may be a practical means of further favoring the growth or survival of an introduced bacterium.

Members of the genus *Pseudomonas* may be especially appropriate for inoculation because some are known to influence the development of higher plants (6) or to antagonize plant pathogens (2). However, other microorganisms might prove to be of greater practical value; for example, those that increase the availability of nitrogen or phosphorus to plants, produce growth regulators, or act detrimentally on plant pathogens. Bacteria that enhance plant growth are not difficult to obtain (12).

Mancozeb is known to decrease the total number of bacteria in soil (4). The fungicide is not known to be extensively metabolized by microorganisms in soil, but it is converted to the active principle, ethylenethiuram monosulfide, which is itself persistent in soil (11, 21). Although mancozeb is useful for the purposes described herein, other fungicides or antibiotics might be appropriate candidates for suppressing the rhizosphere flora. Thiram has already been used for this purpose (14, 16).

The approach described herein has major limitations, however. Among the chief limitations are the lack of appreciable movement of bacteria from the seed coat to distant portions of the root system, the restricted movement of many organic toxicants from the point of their introduction into soil, and the high cost of fungicides and antibiotics. The problem of lack of movement of the inoculum organism from the seed could be alleviated by using an organism that persists from year to year; in this way, the colonists of the distal roots would be cells derived from an inoculum added in a previous season. The restricted movement of many chemicals from seeds to roots or downward after foliar application could be overcome by selecting chemicals showing basipetal translocation; such chemicals are known. Finally, although fungicides and antibiotics are admittedly expensive, the compounds that are selected to enhance root colonization might be those that are already applied to seeds or foliage for disease control.

This approach to enhance colonization is thus potentially useful for increasing plant growth. Further work is needed, however, to define which processes should be enhanced, determine which organisms are most useful for the purposes described, find chemicals that will have the needed properties, and devise means to alter the physiology of the plant to enhance further the beneficial action of the inoculated microorganism.

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