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Intact Soil-Core Microcosms for Evaluating the Fate and Ecological Impact of the Release of Genetically Engineered Microorganisms

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Intact soil-core microcosms were studied to determine their applicability for evaluating the transport, survival, and potential ecosystem effects of genetically engineered microorganisms before they are released into the environment. Soil-core microcosms were planted with wheat and maize seeds and inoculated with *Azospirillum lipoferum* SpBr17 and SpRG20a Tn*5* mutants, respectively. Microcosm leachate, rhizosphere soil, plant endorhizosphere, insects, and xylem exudate were sampled for *A. lipoferum* Tn*5* mutant populations. *A. lipoferum* Tn*5* populations, determined by most-probable-number technique-DNA hybridization, varied from below detection to 10^6 g of dry root^{-1} in the rhizosphere, with smaller populations detected in the endorhizosphere. Intact soil-core microcosms were found to maintain some of the complexities of the natural ecosystem and should be particularly useful for initial evaluations of the fate of plant-associated genetically engineered bacteria.

Methods are needed to evaluate genetically engineered microorganisms (GEMs) in representative ecosystems to assess their fate and potential effects before they are released into the environment. Laboratory-contained microcosms have potential as risk assessment tools prior to field testing. Current philosophy in regard to the release of GEMs into the environment is that each organism should be regulated on the basis of the risk posed by that individual organism, rather than by how that organism was altered (17). A standard test system must be adaptable to evaluate the release of a wide variety of microorganisms as well as detect possible detrimental effects on the ecosystem. Microcosms serve these purposes and can also be used to determine the efficacy of GEMs without the regulatory constraints of field testing. Intact soil-core microcosms have been used to study ecosystem stability (29), nutrient cycling (28), and the effect of utility wastes or other toxic substances on ecological processes (7, 26a, 27). Microcosms have also facilitated the study of microorganism-pollutant interaction in aquatic (20) and sediment ecosystems (9). Whereas chemical substances are dispersed, diluted, or degraded following their introduction into the ecosystem, microorganisms can reproduce and increase in population under favorable conditions. Therefore, microorganisms have the potential to spread and colonize habitats that may be unique to each genotype.

The transposon Tn*5* was used to mutagenize the *A. lipoferum* strains to enhance their detection and enumeration in the microcosms. The presence of Tn*5* in the bacterial genome confers kanamycin resistance (11), which can be used as a selective marker and also as a unique DNA sequence to positively identify mutants by DNA hybridization with a Tn*5*-specific probe (5). In addition, transposon mutants were chosen for these studies because they have been widely used to produce mutants for studying bacterial symbiosis with plants (22), for plant-pathogen interactions (3, 4, 13), and for determining bacterial survival in soil (25). This study was initiated to determine the applicability of intact soil-core microcosms for evaluating GEM transport through the ecosystem, colonization of the rhizosphere and endorhizosphere, displacement of other indigenous rhizosphere microorganisms, and effects on plant nutrient uptake.

MATERIALS AND METHODS

Bacterial cultures, media, and maintenance. *Escherichia coli* MV12, containing the suicide plasmid pGS9 (23), was used as the Tn*5* donor in matings. *A. lipoferum* SpRG20a and SpBr17, ATCC 29708 and 29709, respectively, were purchased from the American Type Culture Collection, Rockville, Md. Luria-Bertani (LB) broth (15), containing 25 μg of kanamycin sulfate ml^{-1} (Sigma Chemical Co., St. Louis, Mo.) and of chloramphenicol ml^{-1} (Sigma) was used for maintenance of *E. coli* MV12. *Azospirillum* cultures were maintained on *Azospirillum* medium (AM), which consisted of nitrogen-free medium (R. Cote, ed., ATCC media handbook, 1st ed., p. 42. American Type Culture Collection, 1984) supplemented with 1.0 g of NH_4Cl and 1.0 g of yeast extract (Difco Laboratories, Detroit, Mich.) liter^{-1}. *Azospirillum* Tn*5* mutants were routinely cultured on AM supplemented with 25 μg of kanamycin sulfate ml^{-1}. Stock cultures of all bacteria were maintained at −80°C in 30% glycerol.

Transposon mutagenesis of *A. lipoferum* strains and physical characterization of mutants was conducted by the methods described by Vanstockem et al. (26). Repeated transfer of the different *A. lipoferum* Tn*5* mutants on N-free AM indicated that the mutants were able to fix N_2 and grow at the same rate in pure culture as the wild type.

Microcosms and inoculation. Intact soil cores for use as microcosms were obtained as described by Van Voris (26a), with a steel coring apparatus containing polyethylene pipe (Phillips Petroleum Co., Dallas, Tex.). The intact soil cores, held inside the plastic pipe after removal from the driving tube, were placed in a housing on Büchner funnels (24-cm-diameter polypropylene funnel [Dynalab Corp., Rochester, N.Y.] or 20-cm-diameter porcelain funnel [Coors, Golden, Colo.]). Two soil-plant combinations common to agricultural regions of eastern Washington were used. Cores (17.8 by 60 cm) of Palouse silt loam soil (fine-silty, mixed, mesic Pachic Ultic Haploxeroll) from the Palouse region were planted with spring wheat (*Triticum aestivum*), while cores (25 by 60

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