Validation of Microcosms for Examining the Survival of *Pseudomonas aureofaciens* (*lacZY*) in Soil

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Evaluating the safety and efficacy of a recombinant bacterium prior to its release into the terrestrial environment requires that risk assessment data be collected in the laboratory. Much of this information is obtained with the use of microcosms. The design of the microcosm significantly affects the ability of the recombinant microorganism to survive in soil and, thus, complicates the risk assessment process. To standardize microcosms for future use, we evaluated the survival of *Pseudomonas aureofaciens* 3732 RN-L11 (*lacZY* Rif^r Nal^r) in intact soil cores (5.0 by 15 cm; polyvinyl chloride core) and disturbed soil microcosms (50 g of fresh, sieved soil). Survival data were compared with those obtained during a field release. The intact soil core microcosm was shown to closely simulate results obtained in the field. The intact soil core microcosm closely predicts survival in bulk soil and in the rhizosphere of wheat. Data obtained with the microcosm were also similar when evaluated in separate studies in two different years. In 1993, *P. aureofaciens* survived for approximately 63 days in bulk soil and 96 days in the rhizosphere. The disturbed soil microcosm exhibited a much more rapid decline in population size (34 days to zero) than the intact core microcosm. We speculate that drying and sieving of soil for the disturbed soil microcosm affected the ability of the soil to support the survival of *P. aureofaciens*. These results demonstrate that a small, inexpensive, and simple intact soil core microcosm may be appropriate for risk assessment.

The impending release of numerous genetically engineered microbes (GEMs) into the terrestrial environment requires that regulators assess the risks associated with each release. Risk assessment is composed of two major components—hazard and exposure. Hazard is a function of the organism and is related to its ability to cause negative effects on humans, animals, or the ecosystem (17). Exposure is more difficult to define but has generally been limited by conducting releases into very confined environments. Before introduction into the field, methods are required to assess survival and competitiveness.

Microcosms are often used to gather the requisite risk assessment data. Microcosms, although uncoupled from nature (12, 23), represent a complex system containing the ecological network from which an organism was obtained. As early as 1976, the Environmental Protection Agency recognized the need for “effective, reliable, rapid, and relatively inexpensive tools and techniques to screen and identify those chemicals having potentially harmful health and ecological effects” (22).

Many different designs of microcosms are available. Design complexity ranges from a few grams of soil in a bottle (15) to a large chamber where many of the environmental variables are controlled (10, 11). Many design variations were summarized in an Environmental Protection Agency workshop on microcosms (6). Fourteen different microcosm designs were identified in what the authors concede is an incomplete list. Unfortunately, the design of a microcosm may significantly affect the results obtained. Obviously, 10 g of dried and sieved soil in a small vial will produce results different from those obtained with a much larger volume of undisturbed soil. Richaume et al. (21) have demonstrated that soil structure is critical for simulating the ability of a GEM to survive in a field environment.

Because the design of a microcosm affects the survival of a GEM in soil and so many microcosm designs are currently utilized, it is nearly impossible to compare data from one study with those from another. This has greatly complicated the decisions made by risk assessors. To begin the process of developing a standard microcosm design, the study described here was initiated. Of the many microcosm designs in use, two were selected that were low in cost and simple to use.

MATERIALS AND METHODS

Microorganisms. *Pseudomonas aureofaciens* 3732 RN-L11 (*lacZY*) was used for all studies. This microorganism, isolated from the rhizosphere of wheat, was previously described by Barry (2, 3). The *lacZY* genes, located on the chromosome, are a highly stable marker for tracking bacteria in soil (1a) and have been reviewed in detail by Drahos et al. (9). *P. aureofaciens* 3732 RN-L11 is also resistant to rifamycin SV (100 µg/ml) and nalidixic acid (100 µg/ml). The microorganism was maintained in *Pseudomonas* F broth (Difco, Detroit, Mich.) with 50% glycerol at –55°C.

An inoculum for addition to soil was prepared by adding a single loopful of *P. aureofaciens* RN-L11 to 10 ml of *Pseudomonas* F broth containing rifamycin SV and nalidixic acid. The culture was grown to an approximate density of 10⁹ CFU ml^{–1}. A small amount of broth was then transferred to 1.5 liters of broth and shaken for approximately 20 h, resulting in a density of 10⁹ CFU ml^{–1}. Prior to inoculation into soil, the culture was centrifuged at 4°C and 5,000 × g for 15 min to pellet cells. The pellet was then resuspended in distilled water to the desired concentration.

Soil. Soil used for all studies was an Adelphia sandy loam collected from the University of Maryland Central Maryland Research and Education Center in Calverton. All soil was obtained from the top 15 cm of the soil profile. Chemical, physical, and biological properties of the soil are presented in Table 1. Notably, the soil contained no bacteria that were resistant to the two antibiotics, grew on *Pseudomonas* F agar, and expressed *lacZY*.

Microcosms. A polyvinyl chloride pipe (15 by 5 cm [inside diameter]) was driven into soil and gently removed by hand. Soil filled all but the top 2 cm of each core. These cores are subsequently referred to as intact soil core microcosms. A polyester mesh membrane (ca. 160-µm mesh; Tetko, Inc., Briarcliff Manor, N.Y.) was placed over the bottom of the core to hold in soil yet allow exchange of gasses and movement of solutes. Cores were immediately placed into a cooler and returned to the laboratory, where they were placed in a refrigerator at 4°C. Microcosms were inoculated within 24 h after collection. One half of the intact soil core microcosms were seeded with three seeds of winter

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TABLE 1. Characterization of field soil into which *P. aureofaciens* 3732 RN-L11 was released

Parameter	Unit	Mean ^a	SD
pH	1:1	5.9	0.16
Cation exchange capacity	mmol kg ⁻¹	7.3	0.87
Organic matter	%	1.77	0.46
N	%	0.15	0.016
C	%	0.97	0.31
Mg	kg ha ⁻¹	372	96
P	kg ha ⁻¹	48	5.4
K	kg ha ⁻¹	290	82
Ca	kg ha ⁻¹	1,267	184
Cu	kg ha ⁻¹	0.78	0.078
Zn	kg ha ⁻¹	4.82	0.69
Mn	kg ha ⁻¹	150	19
Soil H ₂ O content at $-\frac{1}{3}$ bar	%	19.18	1.29
Background microbial population ^b	No. g of dry soil ⁻¹	0	0
Indigenous pseudomonads ^c	No. g of dry soil ⁻¹	$1.70 \cdot 10^5$	$4.10 \cdot 10^3$
Total bacteria	No. g of dry soil ⁻¹	$6.09 \cdot 10^7$	$2.11 \cdot 10^7$
Total fungi	No. g of dry soil ⁻¹	$5.04 \cdot 10^4$	$9.95 \cdot 10^3$

^a Mean of nine replicates.^b Number of indigenous soil bacteria on *Pseudomonas* F agar with antibiotics and X-Gal.^c Number of indigenous pseudomonads on *Pseudomonas* F agar without antibiotics.

wheat (*Triticum aestivum*). Following germination, seedlings were thinned to one plant per core.

Disturbed soil microcosms consisted of 50 g of soil placed into 160-ml milk dilution bottles. Immediately after the soil was collected from the field and before it was placed into bottles, it was air dried and gently passed through a sieve with 0.5-cm openings. Disturbed soil microcosms were not planted with wheat. Microcosm studies were done in both 1992 and 1993.

Following inoculation, the microcosms were placed into a growth chamber and maintained at 28°C and 70% relative humidity day and night. The photoperiod was 12 h with >400 microeinsteins of photosynthetically active radiation m⁻² s⁻¹ from a combination of cool-white fluorescent and incandescent lamps. Soil moisture in all laboratory treatments was maintained at -0.33 bar.

Field plots. Environmental Protection Agency approval was required and granted for release of *P. aureofaciens* 3732 RN-L11 into the field. One-square-meter plots were established 6 months prior to inoculation by killing all vegetation on the area. No pesticides were subsequently applied, and occasional weeds were removed by hand. A 15-cm-high plastic berm was placed around each of the plots to control runoff. Plot design was completely randomized; each plot was then split into two. One half of each plot was seeded to wheat with 10-cm row spacing.

In addition, uninoculated microcosms and plots were examined at each sampling time to confirm the lack of background microorganisms. At all times, no indigenous microorganisms were observed that were resistant to the antibiotics, were able to grow on *Pseudomonas* F agar, and expressed *lacZY*.

Inoculation. Microcosms without wheat were inoculated with *P. aureofaciens* 3732 RN-L11 by dribbling the culture evenly onto the soil surface. Soils were inoculated to a concentration of 10^7 CFU/g of soil. Field plots without wheat were sprayed with the inoculum to achieve a concentration of 10^7 CFU/cm² of soil surface. Wheat plants in the field and microcosms were inoculated by pipetting 1 ml of the inoculum directly onto the crown area of each plant.

Enumeration. All soil within each microcosm was removed, screened to remove rocks and other debris, and thoroughly mixed. Ten grams of soil was then added to 95 ml of dilution buffer (1% CaCl₂) and shaken for 10 min on a rotary shaker (25). Soil was allowed to settle from solution for 10 min. Appropriate dilutions were then spread on *Pseudomonas* F agar containing nalidixic acid (100 µg/g), rifampin SV (100 µg/g), cycloheximide (100 µg/g), and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal; 65 µg/ml). X-Gal is an analog of lactose, and colonies expressing *lacZY* appear blue. Plates were incubated at 28°C for 2 days, and then colonies were counted. Numbers were expressed as CFU per gram of fresh soil. The minimum level of detection was 10 CFU g⁻¹ of soil.

When a root system was present in the microcosms, the rhizosphere was analyzed. Roots were gently separated from the bulk soil and placed into 99 ml of 1% CaCl₂. The mixture was shaken for 10 min, and the soil was allowed to settle from solution for 10 min. The solution was then assayed as described above. Roots were removed from the dilution bottle, dried at 80°C for 12 h, and weighed. Bacterial numbers were expressed as CFU per plant. This method of expression has previously been shown to be the most appropriate for describing risk assessment data (1). Other methods (CFU per gram of soil and CFU per gram of root) are less appropriate, since the reported population is highly dependent upon the size of the root or adhesion of soil to the root.

Soil was removed from the top 15 cm of the field with a 2.5-cm-wide soil core. Five cores per plot were randomly collected and composited into a single sample.

The sample was immediately returned to the laboratory and sieved and mixed, and bacteria were enumerated as before. When wheat was present, the root system was removed with a hand trowel, placed in a plastic bag, and returned to the laboratory and bacteria were enumerated as described above.

Statistics. Laboratory and field experiments were conducted with four replications in a completely randomized design. Analysis of variance was conducted on all data for each day by using log-transformed numbers of CFU to determine significant differences among treatments. Analysis of variance was also conducted for data combined over experiments and days to test for the significance of the linear effect of days and whether the linear effect of days was significantly different in the field and laboratory microcosms. This analysis was used to compare the decline in numbers of CFU between all treatments. Linear regression analysis was used to estimate the rate of decrease in the log number of CFU over time. Linear regression equations were used to compare treatments and to predict the time for the number of CFU to decline to zero.

RESULTS

The use of *lacZY* as a genetic marker proved to be extremely useful for detecting very low numbers of *P. aureofaciens* in soil. Uninoculated soils contained approximately 100 Rif^r and Nal^r CFU (that grew on *Pseudomonas* F medium) per g; however, colonies were not blue and thus were excluded from counting.

Since the number of cells inoculated into soil may vary from experiment to experiment, it is difficult to compare treatment means at each time point. An alternative procedure and one that was highly effective was to statistically compare the slopes of the predicted lines (Table 2). This also allowed us to predict the time in which the population approached zero.

The population of *P. aureofaciens* in the 1993 disturbed soil microcosm declined at a significantly greater rate than in the field or intact core microcosm (Fig. 1). In the disturbed soil microcosm, the population was no longer detectable in soil after 34 days while the population in the intact core was not detectable at 63 days.

The slope of the population decline in the intact soil core microcosm was nearly identical to the slope observed in the field. There was an approximately 0.5-log difference in the population at every time point, including the predicted days to zero. This difference was due to the difference in the amount of inoculum added at time zero. Although we were careful to add similar amounts of inoculum to both the microcosms and field plots, the inoculation methods made this difficult. Nonetheless, slopes were not significantly different, indicating that the intact core was an ideal tool for predicting survival in the field.

TABLE 2. Linear regression of log-transformed numbers of GEM (*P. aureofaciens* 3732 RN-L11) CFU in soil

Treatment	Yr	Slope	Slope SE	Lower 95% confidence interval	Upper 95% confidence interval	y intercept	No. of days to zero (x intercept)
Laboratory rhizosphere	1992	-0.035	0.004	-0.045	-0.026	6.724	190
Laboratory rhizosphere	1993	-0.064	0.013	-0.093	-0.036	6.169	96
Field rhizosphere	1993	-0.055	0.009	-0.074	-0.036	6.428	117
Laboratory intact core	1992	-0.103	0.003	-0.110	-0.096	7.183	70
Laboratory intact core	1993	-0.089	0.005	-0.100	-0.078	5.579	63
Field soil	1993	-0.089	0.009	-0.109	-0.069	5.011	56
Laboratory disturbed soil	1993	-0.208	0.009	-0.226	-0.189	6.958	34

The *P. aureofaciens* populations in intact soil cores in 1992 and 1993 were compared (Fig. 2). The slopes of decline were not significantly different, and the numbers of days to zero were very similar.

Similar studies were repeated with core microcosms and the field by examining the population in the rhizosphere. The specific population within the rhizosphere was not measured until 7 days after the original inoculation. Before this time, the root system was too small to be accurately sampled. At all sampling times throughout the duration of the study, populations in the rhizosphere of wheat in the intact core microcosm and the field were statistically identical (Fig. 3). Further, the slopes over time were not statistically significantly different (Table 2).

P. aureofaciens was originally isolated from the rhizosphere and is therefore well adapted for survival in this region. The GEM survived approximately twice as long in the rhizosphere as in bulk soil (Fig. 1 and 3). In 1993, *P. aureofaciens* could not be isolated after approximately 63 days in the intact core microcosm and after 96 days in the rhizosphere. To some extent, this difference was related to the continual development and colonization of new roots. Since the population in the rhizosphere is expressed as CFU per plant, as opposed to CFU per g of root as in many other studies, we measured the total

population within the rhizosphere as opposed to the concentration within this area.

Results obtained with core microcosms in 1992 and 1993 were also compared (Fig. 4). As noted, when expressed as CFU per plant, *P. aureofaciens* survived significantly longer in 1992 than in 1993. The slopes of decline for the two years were significantly different. The lack of similarity can be partially explained by the poor growth of the wheat in 1993. To ensure uniformity between years, the same lot of seed was used, and between years, the seed was stored at 4°C. Following storage, the seed germination and early growth in 1993 were significantly poorer than in 1992. Since results are expressed as CFU per plant, the size of the root system is an important variable controlling the population in the rhizosphere. When results were normalized to remove root size as a variable and the population was expressed as CFU per g of root (as is typically done in many studies [7, 8, 16]), we found that the rhizosphere populations were more similar for the two years (data not shown).

DISCUSSION

Destruction of aggregates by partial sieving and drying alters the soil so as to limit the survival of bacteria. Richaume et al.

FIG. 1. CFU of *P. aureofaciens* 3732 RN-L11 in soils during 1993. FIG. 2. CFU of *P. aureofaciens* 3732 RN-L11 in soil contained in intact core microcosms.

FIG. 3. CFU of *P. aureofaciens* 3732 RN-L11 in rhizosphere (rhiz.) soils.

(21) have shown that when recombinant organisms are inoculated into soil, they become established within aggregates. Organisms that do not reside within aggregates die much more rapidly. Since many studies (13, 18) have previously used soil that was dried and sieved, die-off rates fail to simulate actual field conditions, as demonstrated in the present study. For example, Naik et al. (18) examined survival and gene transfer of soil microbes in soil that had been previously dried, ground with a mortar and pestle, passed through a 0.242-mm sieve, and sterilized. The microcosm consisted of 2 g of soil in a test

FIG. 4. CFU of *P. aureofaciens* 3732 RN-L11 in rhizosphere (rhiz.) soils.

tube. It is therefore important to maintain the structural integrity of soil during evaluation of microbial survival.

Drying of soil prior to addition to microcosms causes many chemical properties of the soil to be altered (24). Specifically, drying releases a number of inorganic ions, such as manganese, that may be toxic to the bacterium. Several groups of researchers have recognized the inherent problems associated with the use of dried and sieved soil and have utilized only soil that has been maintained moist following collection and passed through a relatively coarse sieve to remove only large debris (14, 19, 20).

The advantage of using dried and sieved soil is that much of the spatial variability associated with intact soil is removed. Coefficients of variation associated with the disturbed soil microcosm were lower than that associated with the field or core microcosms (data not presented). For predictive purposes, however, the variation with the intact core microcosm was not excessive and the small increase in variation was outweighed by other advantages of the intact core microcosm. Microcosms using small quantities of dried and sieved soil may also be suitable for examining relative differences between organisms or soils. In such studies, similarity to survival in the field is not of paramount importance.

Other studies have used extremely complicated microcosms in which many of the environmental variables were controlled and much of the inherent soil structure was maintained. These microcosms may realistically simulate conditions in the field; however, their high cost and the difficulty of manipulating soil and treatments may limit their usefulness. Further, the inability to replicate large microcosms reduces the number of variables that can be examined in any single study.

The current study has shown that a simple and cost-effective microcosm is adequate for providing the necessary information required by risk assessors considering a potential field release. The intact core microcosm is extremely inexpensive and simple to utilize. Cost and complexity are limiting factors that affect acceptance. These factors are important since tests may be required to utilize many soils under a variety of conditions.

Larger, yet similar, intact soil core microcosms were previously utilized for evaluating survival and gene transfer (4, 5). These microcosms were utilized for both bulk soil and rhizosphere soil and were found to be effective for predictive purposes compared with survival in the field. While large core microcosms greatly increase the complexity associated with each of the studies, the size permitted evaluation of such factors as leaching through the soil profile.

Although it has been shown that the intact core microcosm is effective for predicting the survival of *P. aureofaciens* in a single Maryland soil, much work is required before it can be accepted as a standard risk assessment tool. Validation with additional soils and other organisms is necessary prior to acceptance.

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