Effect of Repetitive Applications of the Biocontrol Bacterium *Pseudomonas putida* 06909-rif/nal on Citrus Soil Microbial Communities

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ABSTRACT

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The effects of repetitive applications of *Pseudomonas putida* 06909-rif/nal on the resident microbial communities within a citrus orchard were studied with fatty acid methyl-ester (FAME) profiles and ribosomal intergenic spacer analysis. The data set from FAME was large and very complex, requiring 23 factors from principal component analysis to explain 91% of variability in the data. Spatial and temporal effects on variation within microbial communities were much greater than the

effects of either yearly applications of *Pseudomonas putida* 06909-rif/nal, weekly repetitive applications of *Pseudomonas putida* 06909-rif/nal, or yearly applications of the fungicide metalaxyl and the nematocide phenamiphos. Multivariate analysis of covariance showed much of the variability between treatments could be accounted for by populations of *Pseudomonas putida* 06909-rif/nal. Soil fatty acids that showed significant changes between treatments were not related to fatty acids found in *Pseudomonas putida* 06909-rif/nal, suggesting applications of *Pseudomonas putida* 06909-rif/nal altered the soil microbial community.

Additional keywords: inundative biological control, microbial ecology.

Biological control has been proposed for the replacement of chemical control of plant diseases or for control of diseases that are not economically controlled with chemicals (3,7,14,15,20,24,27). Biological control of plant pathogens has been observed as a natural phenomenon (2), lending credence to this concept. A great deal of effort has gone into elucidating the mechanisms of biological control and some efforts have been fruitful (2,9,13,19,26,32,41). Unfortunately, few commercial biocontrol products for the control of soilborne plant pathogens are being successfully marketed (3,13,14). While biological control has been effective and widely accepted in entomology and weed science, biological control of soilborne plant pathogens has encountered several obstacles that are just now being overcome. Some of these obstacles led Schroth and Weinhold (27) to call rhizosphere investigations a "masochist's delight."

Many researchers have suggested that insufficiencies in biological control may be overcome through inundative means (7), but few have attempted this due to the high cost of applications. Bahme et al. (1) showed that repetitive applications of *Pseudomonas fluorescens* through a drip-irrigation system improved colonization of the root system of potatoes over seed piece inoculation or incorporation of bacterial-impregnated granules into the soil. Eco Soil Systems (San Diego, CA) developed a self-contained field fermentor, called the BioJect, to overcome the costs and difficulties inherent in repetitive applications of bioinoculants. The BioJect is capable of producing 120 liters of high-density bacterial inoculum under field conditions (30,31).

Pseudomonas putida 06909 is an effective biocontrol agent of *Phytophthora parasitica* and *Phytophthora citrophthora*. *Pseudo-*

monas putida 06909 was originally isolated as a contaminant on a field isolate of *Phytophthora citrophthora*. *Pseudomonas putida* 06909 can effectively reduce populations of *Phytophthora citrophthora* and *Phytophthora parasitica*, the causal agents of *Phytophthora* root rot of citrus, in greenhouse studies and *Phytophthora citrophthora* in seedbed trials (34,35). It actively adheres to the hyphae of *Phytophthora* sp. and restricts growth of the pathogen in vitro. It produces an iron chelating siderophore, pyoverdine, but shows no evidence of antibiosis (36,42).

Field trials reported in Steddom et al. (30) resulted in large numbers of bacteria being applied to the soil in an effort to control populations of *Phytophthora parasitica* and the resulting disease, *Phytophthora* root rot of citrus. The addition of large numbers of organisms can have wide-ranging impacts on the resident community. Lynch (18) stressed the importance of studying possible effects of introduced organisms on the environment and nontarget organisms. Simberloff and Stiling (28) cited several examples of introduced vertebrate, invertebrate, and viral agents impacting nontarget organisms. In a rebuttal to Simberloff and Stiling, Frank (11) argued the importance of weighing the risk of an introduced organism against the possible benefit as well as considering the risks associated with alternative methods such as chemical control.

Cook et al. (8) identified four safety issues related to unintended adverse effects of microbial biocontrol agents: (i) competitive displacement, (ii) human allergenicity, (iii) toxigenicity of metabolites, and (iv) pathogenicity. Weller (40) found that introductions of *Pseudomonas fluorescens* 2-79 increased the number of fluorescent pseudomonads in the soil over control soils, but did not impact total numbers of bacteria. Gilbert et al. (12) found that coating seeds with a strain of *Bacillus cereus* had dramatic effects on the resident spermosphere community in two experiments but had little effect in two other experiments. It was unclear from the results whether the observed differences

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were due to the biocontrol agent as an active member of the community or if the biocontrol agent served as a food source utilized by the community when the biocontrol agent failed to persist. Natsch et al. (22) found carbon source utilization was affected more by the sampling date than by inoculations with *Pseudomonas fluorescens*.

All of these studies suffer from difficulties in assessing the microbial community. As early as 1932, Waksman (38) noted “numerous soil organisms develop upon artificial media only with great difficulty and are often repressed there by certain types which may be only occasional visitors in the soil.” Recently, new techniques have revealed just how correct Waksman was. Torsvik et al. (33) used DNA reassociation curves to estimate that over 4,000 species of bacteria were present in a single gram of soil. Sequencing of the small ribosomal subunit has been used to identify organisms that cannot be easily cultured, revealing previously unknown groups of microorganisms (39). This has revolutionized our current understanding of bacterial taxonomy as well as opened new perspectives on microbial ecology (16,23). Lipid analysis has also been used to study the taxonomy of organisms and has been adapted to microbial ecology (6,37). Neither lipid analysis nor nu-

cleic acid analysis requires that organisms be cultured to be identified, provided that enough nucleic acid or lipids can be extracted. It is unclear how fatty acid profiles will relate to DNA profiles. In a DNA profile, each band represents a single organism or a group of organisms. Each organism however has multiple fatty acids, so a fatty acid profile cannot be related back to single organisms in a predictable fashion. Combination of new techniques with traditional microbiological methods provides a better picture of the total complexity of soil microbial communities and how they are impacted by various factors.

The purpose of this study was to determine the impact of the continuous application of the biocontrol bacterium *Pseudomonas putida* 06909-rif/nal on the resident soil microbial community and to assess its impact in relation to other sources of variation such as season, climate, and spatial variability.

MATERIALS AND METHODS

Field plot. A field plot was established at the University of California Lindcove field station in California's Central Valley as previously described in Steddom et al. (30). The plot was located

TABLE 1. Number and percentage of samples each was fatty acid detected in and univariate statistics from fatty acid methyl-esters data from all 3 years

Fatty acid	Count	Percent	Sampling date			Block			Treatment		
			df	F	P > F	df	F	P > F	df	F	P > F
9:0	7	3	8	2.27	0.0267	4	0.74	0.5639	2	5.17	0.0070
12:0	232	86	8	5.01	<0.0001	4	9.69	<0.0001	2	31.68	<0.0001
C9 Dicarboxylic acid	187	69	8	2.36	0.0215	4	26.05	<0.0001	2	16.32	<0.0001
11:0 3OH	9	3	8	7.89	<0.0001	4	2.45	0.0498	2	0.97	0.3813
12:1 3OH	8	3	8	7.28	<0.0001	4	2.66	0.0357	2	0.53	0.5892
14:0	270	100	8	3.07	0.0035	4	7.21	<0.0001	2	6.18	0.0028
13:0 ISO 3OH	21	8	8	29.75	<0.0001	4	2.95	0.0230	2	0.01	0.9894
15:0 ISO	269	100	8	4.28	0.0001	4	2.00	0.0990	2	0.70	0.4993
15:0 ANTEISO	236	87	8	3.51	0.0011	4	6.94	<0.0001	2	4.41	0.0142
15:1 w7c	29	11	8	49.78	<0.0001	4	3.63	0.0080	2	0.21	0.8076
16:0 ISO	246	91	8	2.17	0.0342	4	5.75	0.0003	2	4.80	0.0098
16:1 w9c	9	3	8	7.45	<0.0001	4	2.22	0.0707	2	0.21	0.8080
16:1 w7c	216	80	8	5.41	<0.0001	4	5.31	0.0006	2	0.16	0.8554
16:1 w5c	195	72	8	4.95	<0.0001	4	8.34	<0.0001	2	1.08	0.3433
16:1 w3c	12	4	8	6.70	<0.0001	4	4.20	0.0032	2	0.12	0.8909
16:0	270	100	8	13.82	<0.0001	4	3.03	0.0202	2	0.35	0.7057
17:1 Alcohol (w8)	7	3	8	5.52	<0.0001	4	2.65	0.0365	2	0.30	0.7439
ISO 17:1 G	193	71	8	4.87	<0.0001	4	20.76	<0.0001	2	2.75	0.0678
15:0 3OH	5	2	8	2.24	0.0292	4	2.04	0.0936	2	0.99	0.3744
17:0 ISO	36	13	8	11.35	<0.0001	4	2.27	0.0658	2	1.19	0.3067
17:0 ANTEISO	139	51	8	8.09	<0.0001	4	0.64	0.6366	2	0.28	0.7553
18:1 w9c Alcohol	11	4	8	9.44	<0.0001	4	0.82	0.5119	2	1.24	0.2944
18:1 w9t Alcohol	21	8	8	14.82	<0.0001	4	1.05	0.3849	2	1.81	0.1674
18:3 w6c	58	21	8	3.48	0.0012	4	2.63	0.0379	2	0.65	0.5230
18:2 w6c	270	100	8	4.37	0.0001	4	3.59	0.0084	2	11.36	<0.0001
18:1 w9c	270	100	8	6.17	<0.0001	4	3.17	0.0163	2	2.25	0.1094
18:1 w8t	5	2	8	0.83	0.5815	4	0.94	0.4424	2	0.96	0.3861
18:0	262	97	8	6.04	<0.0001	4	1.42	0.2310	2	1.30	0.2771
19:2 w6c	31	11	8	19.99	<0.0001	4	3.31	0.0131	2	0.00	0.9983
19:1 w8t	6	2	8	1.34	0.2292	4	0.79	0.5364	2	0.25	0.7785
19:0 cyclo c11-12	9	3	8	1.79	0.0852	4	0.87	0.4812	2	0.66	0.5207
20:3 w6c	9	3	8	4.85	<0.0001	4	1.50	0.2074	2	3.69	0.0278
20:4 w6c	6	2	8	1.48	0.1732	4	0.62	0.6485	2	3.33	0.0391
Sum in feature 4 ^a	18	7	8	0.46	0.8836	4	1.43	0.2287	2	0.77	0.4667
cis 9,10 epoxy 18:0	30	11	8	11.69	<0.0001	4	3.02	0.0206	2	0.10	0.9045
Sum in feature 8 ^b	266	99	8	14.20	<0.0001	4	10.94	<0.0001	2	0.82	0.4436
21:0 ISO	162	60	8	3.99	0.0003	4	1.62	0.1746	2	0.27	0.7604
Sum in feature 11 ^c	267	99	8	4.68	0.0001	4	1.16	0.3323	2	5.06	0.0078
C18 N Alcohol	116	43	8	7.19	<0.0001	4	29.09	<0.0001	2	1.28	0.2824
C22 Primary alcohol	121	45	8	5.85	<0.0001	4	2.52	0.0448	2	5.63	0.0046
22:1 w9c	85	31	8	30.70	<0.0001	4	8.61	<0.0001	2	0.43	0.6518
23:1 w7c	8	3	8	7.08	<0.0001	4	2.02	0.0961	2	0.76	0.4699
C25 N Alcohol	7	3	8	1.05	0.4026	4	2.25	0.0681	2	0.14	0.8706
25:0	202	75	8	1.20	0.3071	4	0.79	0.5362	2	1.58	0.2095

^a Feature 4 consists of 17:1 ISO and 17:1 ANTEISO, they have similar retention times and cannot be resolved.

^b Feature 8 consists of 18:1 w9t.

^c Feature 11 consists of 19:1 w6c and 19:0 cyclo, they have similar retention times and cannot be resolved.

on the site of a former citrus grove that had a history of *Phytophthora* damage. The soil was a San Joaquin sandy loam with the following characteristics: pH 7.0; electrical conductivity, 0.60 mmho/cm; organic matter, 0.71%; cation exchange capacity, 17.2 meq per 100 g; Ca, 4.1 meq/liter; Mg, 1.0 meq/liter; Na, 0.5 meq/liter; Cl, 0.3 meq/liter; B, 0.1 ppm; soluble Zn, 8.4 ppm; soluble Mn, 35.2 ppm; soluble Fe, 27.0 ppm; Cu, 5.6 ppm; N-total, 0.064%; and soluble K, 16.9 ppm. Nursery-grown navel seedlings on troyer citrange rootstock were arranged in a randomized complete block design with 18 replicate blocks, and a single tree per treatment in each block. The soil under each tree was infested 4 weeks after planting by incorporating 50 g of millet colonized with *Phytophthora parasitica* (M288) into the top 2 to 4 cm of soil and immediately irrigating the trees. Treatments were initiated 12 months after planting. Treatments consisted of (i) a water control, (ii) a yearly application of *Pseudomonas putida* 06909-rif/nal at the beginning of each irrigation season, (iii) repetitive applications of *Pseudomonas putida* 06909-rif/nal with every irrigation, and (iv) a fungicide/nematicide treatment consisting of yearly applications of metalaxyl, Ridomil Gold EC at 2.35 liters/ha, and phenamiphos, Nemaquire at 14.1 liters/ha, diluted in water and applied as a soil drench just prior to irrigation. Applications of *Pseudomonas putida* 06909-rif/nal treatments consisted of 100 ml per tree of a late log phase culture (24 h at 24°C, averaging 2×10^8 CFU/ml over the span of the experiment) applied through a separate irrigation line with a chemical injector to continuous application treatment trees, or by hand as a soil drench, diluted in 10 liters of water and placed around the base of the single application treatment trees just prior to irrigation. The irrigation line was split into two lines and the chemical injector installed on one line after the split, leading to the continuous application treatment trees, and the second line leading to all other treatments. This allowed uniform irrigation practices for all treatments. Two half-circle minisprinklers with a flow rate of 20 liters/h were installed to each side of the base of each tree pointing away from the trunk. Mean populations of *Pseudomonas putida* 06909-rif/nal in the irrigation water were 2×10^6 CFU/ml with a mean dilution rate of 1.5×10^{-3} for a 1-h irrigation. Trees were irrigated once a week during most of the irrigation season, approximately April to November, with times ranging from 30 min to 3 h as needed. At the beginning and end of each irrigation season, irrigations were once every 14 days or as needed. Soil samples for assessment of microbial communities were taken from the control, yearly application treatment, and weekly application treatment trees in July, November, and March of each year, beginning in July 1997 and continuing to March 2000, on 5 blocks randomly selected out of the 18 possible blocks. Sampling time corresponded to early in the irrigation season, end of the irrigation season, and end of the nonirrigated season. In November 1999 and March 2000, samples were also collected from trees receiving yearly applications of the fungicide, metalaxyl, and the nematicide, phenamiphos. The blocks that were selected for sampling were located between 12 and 40 m apart, as measured from the center of one block to the center of another. Samples consisted of four 20- × 2.5-cm soil cores per tree to a depth of 25 cm from the wetted area of the minisprinklers that were pooled and mixed for a composite sample. Samples were placed in an insulated chest and transported back to the lab at ambient temperature. Bacterial populations were determined and reported in Steddom et al. (30). Samples were frozen at -20°C, freeze-dried, sieved through a 1-mm sieve, and thoroughly mixed. Samples were then returned to -20°C until analysis.

Fatty acid methyl-esters. Samples from July 1997 through March 2000 were analyzed by fatty acid analysis. Fatty acid methyl-esters (FAME) were extracted according to the method of Buyer et al. (5), except 2 g of freeze-dried soil was used. To minimize variability and insure against missing samples, each set of extractions was repeated and each sample was duplicated

in each set of extractions. Results for each sample were averaged before statistical analysis. The percentage of each fatty acid in a single sample was used for analysis in principal component analysis, multivariate analysis of variance (MANOVA), and multivariate analysis of covariance (MANCOVA), with soil bacterial populations used as the covariate (SAS Institute, Cary, NC).

Ribosomal intergenic spacer analysis. Samples from November 1999 and March 2000 were analyzed with the ribosomal intergenic spacer analysis (RISA) method of Borneman and Triplett (4). This included samples from the fungicide/nematicide treatment for November and March. DNA was extracted from 500-mg samples of freeze-dried soil using the FastDNA kit for soil (Bio101, Inc., Vista, CA) and resuspended in 100 µl of Tris-EDTA (pH 8.0). RISA was performed according to Borneman and Triplett (4) except where noted. The ribosomal intergenic spacer region was amplified in 100-µl polymerase chain reaction (PCR) with 2.5 µl of soil DNA template. DNA template was denatured for 1 min at 94°C, amplified for 31 cycles of PCR at 94°C for 15 s, 56°C for 30 s, and 72°C for 45 s, followed by extension for 1 min at 72°C. PCR products were purified with a PCR purification kit (QIAquick; Qiagen, Inc., Valencia, CA) and resuspended in 50 µl of 10 mM Tris (pH 8.5). Purified PCR products were denatured at 95°C for 5 min in 95% formamide and resolved on prerun (30 min), 5% polyacrylamide gels for 2.5 h at 55 to 60°C (≈ 49 W). A molecular weight standard, Φ x174/HaeIII (Promega, Madison, WI), was run at both sides of the gel and in the center lane. All samples from a single block were run on a single gel. Gels were prepared and stained with a sequence kit (Promega Silver). Gels were allowed to dry onto the glass plate at room temperature and they were scanned at 800 dpi on a flat bed scanner. Images were straightened in Quantity One (Bio-Rad, Hercules, CA) by aligning the 1,353- and 310-bp bands in the standards. Data were output to a text file. This file gave the densities of each lane according to retardation factors (R_f), in which an R_f of 0 corresponded to 1,353 bp and an R_f of 1 corresponded to 310 bp. Densities in each lane were averaged by intervals of 0.02 R_f units from 0.01 to 0.60 R_f , resulting in 31 segments from 1,343 to 727 bp. This procedure assumed that background staining was uniform across the gel. Densities at each of these regions for each lane were used in MANOVA.

TABLE 2. Eigenvalues, proportion of variance explained, and cumulative variance explained from principal component analysis on fatty acid methyl-esters data from all 3 years

Eigenvector	Eigenvalue	Proportion	Cumulative
1	8.1687	0.1857	0.1857
2	4.5307	0.1030	0.2886
3	3.0426	0.0692	0.3578
4	2.5823	0.0587	0.4165
5	2.0555	0.0467	0.4632
6	1.9334	0.0439	0.5071
7	1.7881	0.0406	0.5478
8	1.6198	0.0368	0.5846
9	1.5436	0.0351	0.6197
10	1.4890	0.0338	0.6535
11	1.3735	0.0312	0.6847
12	1.2853	0.0292	0.7139
13	1.1180	0.0254	0.7392
14	1.0166	0.0231	0.7624
15	0.9426	0.0214	0.7839
16	0.9026	0.0205	0.8044
17	0.8245	0.0187	0.8231
18	0.7468	0.0170	0.8401
19	0.7123	0.0162	0.8563
20	0.6856	0.0156	0.8719
21	0.5909	0.0134	0.8853
22	0.5687	0.0129	0.8982
23	0.5471	0.0124	0.9106

RESULTS

FAME. A total of 80 different fatty acids were detected however many were present in only a few samples. Only fatty acids that were present in more than 1.5% of the samples were used for statistical analysis. This left 44 fatty acids for all 3 years together and 31 fatty acids for the final two sampling dates with the chemical treatment (Table 1). Principle component analysis revealed 12 factors that explained 71% of the variance of the data and 23 factors that explained 91%, indicating a complex community structure (Table 2). MANOVA showed significant variation across sampling times, treatment, and blocks. Sampling times showed the greatest amount of variation, followed by blocks, and then treatment (Table 3). Contrasts showed a significant difference between irrigation year 1997 (July 1997, November 1997, and March 1998) and irrigation year 1998 (July 1998, November 1998, and March 1999), and also between irrigation year 1998 and irrigation year 1999 (July 1999, November 1999, and March 2000). All blocks were different from each other according to contrasts. Likewise, all treatments were different from each other. The MANOVA provides a univariate analysis of variance for each fatty acid in the model. Of the 44 fatty acids included in the analysis, 37 showed significant differences between sampling dates, 24 showed significant differences between blocks, and 10 showed significant differences between treatments (Table 1). Of the 11 fatty acids present in *Pseudomonas putida*, only the four straight-chained fatty acids (12:0, 14:0, 16:0, and 18:0) were detected in the soil (Table 4). Least squares means of these fatty acids showed an increase in the fatty acids 16:0 and 18:0 in the treatments receiving bacterial applications and a decrease in the fatty acids

12:0 and 14:0. When *Pseudomonas putida* populations were used as a covariate in MANCOVA, the effect of different size populations of *Pseudomonas putida* 06909-rif/nal was removed from sampling time, block, and treatment factors. Sampling times and blocks remained significantly different (Table 5). Water controls were no longer significantly different from either the yearly applications of bacteria or from the weekly applications of bacteria. Weekly applications of bacteria were still significantly different from yearly applications of bacteria, although the significance level dropped. MANOVA of the final two sampling times when the chemical treatment was included, showed a significant difference between the two sampling times and blocks, but not between treatments or adjacent blocks (Table 6).

RISA. The ribosomal intergenic spacer region was used to categorize populations from soil microbial communities from November 1999 and March 2000 into 31 operational taxonomic groups with sizes ranging from 1,343 to 727 bp. These profiles of operational taxonomic groups were analyzed with MANOVA and contrasts. None of the treatments, including the chemical treatment, showed detectable differences in the soil microbial community (Table 7).

DISCUSSION

Fatty acid profiles are a very sensitive method for quantifying changes in soil microbial communities (6). We initially found a total of 80 unique fatty acids, even though each soil sample had only an average of 20 fatty acids. Many of these fatty acids were present in a small number of soil samples, and for the purpose of this study, it was decided to discard any fatty acids that were not in at least 1.5% of the samples. However, it is likely that these fatty acids were important components of the microbial communities with these samples, since they were consistent across the multiple subsamples that were analyzed and later averaged. The large number of principal component factors needed to account for

TABLE 3. Multivariate analysis of variance from fatty acid methyl-esters data for all 3 years

	Wilks' Lambda	F	Numerator df	Denominator df	P > F
Sample time	0.0000	5.54	352	625	<0.0001
1997 vs. 1998	0.0598	27.51	44	77	<0.0001
1998 vs. 1999	0.2307	5.84	44	77	<0.0001
Block	0.0135	3.42	176	310	<0.0001
2 vs. 3 (27 m) ^a	0.3782	2.88	44	77	<0.0001
3 vs. 8 (12 m)	0.4953	1.78	44	77	0.0131
8 vs. 18 (24 m)	0.3250	3.63	44	77	<0.0001
17 vs. 18 (27 m)	0.4099	2.52	44	77	0.0002
Treatment	0.2187	1.99	88	154	<0.0001
Water vs. yearly	0.5063	1.71	44	77	0.0200
Water vs. weekly	0.4473	2.16	44	77	0.0015
Weekly vs. yearly	0.4495	2.14	44	77	0.0017

^a Numbers in parentheses are distances in meters between centers of blocks.

TABLE 4. Fatty acids found in *Pseudomonas putida* and least squares means of fatty acids recovered from soil from water control, yearly applications of *Pseudomonas putida*, or weekly applications of *Pseudomonas putida* treatments^a

Fatty acid	Putida	Water	Yearly	Weekly
10:0 3OH	3.97	...	...	...
12:0	2.69	3.14	2.67	1.82
12:0 2OH	4.35	...	...	...
12:0 3OH	4.24	...	...	...
14:0	0.36	5.72	5.32	5.00
16:0	28.44	19.00	19.31	19.25
17:0 CYCLO	3.61	...	...	...
18:1 w7c	19.31	...	...	...
18:0	0.69	3.55	3.84	3.65
19:0 CYCLO w8c	0.41	...	...	...
Sum in feature 3 ^b	31.85	...	...	...

^a ... indicates that the fatty acid was not detected in these samples.

^b Feature 3 consists of 16:1 w7c and 15:0 ISO 2OH, they have similar retention times and cannot be resolved.

TABLE 5. Multivariate analysis of covariance from fatty acid methyl-esters data for all 3 years

	Wilks' Lambda	F	Numerator df	Denominator df	P > F
Sample time	0.0000	5.70	352	601	<0.0001
1997 vs. 1998	0.0456	35.23	44	74	<0.0001
1998 vs. 1999	0.2296	5.64	44	74	<0.0001
Block	0.0140	3.24	176	298	<0.0001
2 vs. 3 (27 m) ^a	0.3813	2.73	44	74	<0.0001
3 vs. 8 (12 m)	0.5014	1.67	44	74	0.0252
8 vs. 18 (24 m)	0.3188	3.59	44	74	<0.0001
17 vs. 18 (27 m)	0.4135	2.39	44	74	0.0005
Treatment	0.2843	1.47	88	148	0.0192
Water vs. yearly	0.5657	1.29	44	74	0.1641
Water vs. weekly	0.5828	1.20	44	74	0.2375
Weekly vs. yearly	0.5058	1.64	44	74	0.0294
Log10CFU	0.6057	1.09	44	74	0.3597

^a Numbers in parentheses are distances in meters between centers of blocks.

TABLE 6. Multivariate analysis of variance from fatty acid methyl-esters data for the final two sampling dates including the chemical treatment

	Wilks' Lambda	F	Numerator df	Denominator df	P > F
Sample time	0.0016	42.86	30	2	0.0230
Block	0.0000	4.75	120	11	0.0040
2 vs. 3 (27 m) ^a	0.0099	6.68	30	2	0.1383
3 vs. 8 (12 m)	0.0176	3.72	30	2	0.2337
8 vs. 18 (24 m)	0.0105	6.28	30	2	0.1464
17 vs. 18 (24 m)	0.0038	17.69	30	2	0.0549
Treatment	0.0001	1.62	90	7	0.2618

^a Numbers in parentheses are distances in meters between centers of blocks.

the variability of the reduced data set provides further proof of the complex nature of these microbial communities.

MANOVA and MANCOVA allowed us to study the relative importance of seasonal effects, location, and treatment independently of each other. Seasonal effects were the largest contributors to the variability of the data. During the 3 years in which this project was conducted, California experienced the El Nino phenomenon in the winter of 1997 and a heavy freeze in the winter of 1998. As sampling time progressed through this study, the trees in question grew from approximately 2 feet to over 6 feet high, with a commensurate increase in root volume. It was surprising to see large differences in microbial communities across a 0.4-ha field. Some of the blocks that were studied were adjacent to each other with a mean distance of only 12 m, yet the communities were different between them. The field was very uniform with no visible topography or differences in soil types. Treatment was consistently the smallest effect in this study. This is in spite of our additions of over 1×10^{12} CFU of *Pseudomonas putida* 06909-rif/nal per tree over a 3-year period. However, considering that typical soils contain between 1×10^8 and 1×10^{11} cells per g of soil, the applications of *Pseudomonas putida* 06909-rif/nal were actually a very small portion of the overall community. Also, *Pseudomonas putida* 06909-rif/nal never reached populations over 1×10^5 CFU/g of soil. Using measured populations of *Pseudomonas putida* 06909-rif/nal as a covariate only removed some of the treatment effect. *Pseudomonas putida* 06909-rif/nal is not known to produce any antibiotics but it is applied with the spent media containing metabolites including an iron chelating siderophore. Either the application of these metabolites or the presence of *Pseudomonas putida* 06909-rif/nal affected the microbial community in ways that could not be accounted for simply by population size. The fatty acids 18:1 w7c and those in feature 3 are present in *Pseudomonas putida* in large quantities, yet they were never detected in the soil. This provides further evidence that the community was altered by applications of the biocontrol agent.

Introduced biocontrol microorganisms can have large or small impacts on microbial communities depending upon their principal mode of action. Microorganisms produce a number of metabolites with different ranges of activity (17). Robleto et al. (25) looked at the effect of a narrow spectrum antibiotic, trifolitoxin, on microbial communities and found no effect on the overall community but strong effects on trifolitoxin sensitive organisms. Although little is known about the mode of action of *Pseudomonas putida* 06909-rif/nal, it inhibited *Phytophthora parasitica* to a small degree (30) but had little or no effect on the citrus nematode *Tylenchulus semipenetrans* (29).

Fatty acid analysis appeared to be the most sensitive technique, but interpretation of fatty acid profiles is difficult because each organism has multiple fatty acids with few specific to any distinct group. Analysis of the ribosomal intergenic region provided a large number of bands that were visually different between treatments but due to gel warping and uneven staining there was no way other than our computer imaging analysis to compare these in an unbiased manner. A preferred technique would have been the automated form of RISA (10), which improves the precision and accuracy of nucleic acid detection with the use of internal standards. An advantage of RISA, as a nucleic acid-based method, is

that it allows for identification of organisms through sequencing of individual bands recovered from the gel. This could allow for more detailed studies of subsets of the community. A second DNA-based analysis, denaturing gradient gel electrophoresis, was attempted but abandoned due to the lack of specific bands identified from bulk soil.

Our results match those of Weller (40) and Natsch et al. (21,22), who found only minor impacts from introduced organisms and questioned whether repetitive inoculations would have an increased effect. While there is a strong impact on survival of the introduced organism, our study suggests repetitive applications of *Pseudomonas putida* 06909-rif/nal have a small impact on soil microbial communities. To our knowledge, this is the first study looking at the impact of large-scale repetitive applications of a organism on the existing microbial community.

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TABLE 7. Multivariate analysis of variance from ribosomal intergenic spacer analysis data for the final two sampling dates including the chemical treatment

	Wilks' Lambda	F	Numerator df	Denominator df	P > F
Sample time	0.0432	1.48	30	2	0.4846
Block ^a	0.0000	4.29	120	11	0.0061
Treatment	0.0004	0.94	90	7	0.6057

^a Block effects include differences between gels, contrasts not examined.

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