Repetitive Applications of the Biocontrol Agent *Pseudomonas putida* 06909-rif/nal and Effects on Populations of *Phytophthora parasitica* in Citrus Orchards

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ABSTRACT

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Pseudomonas putida 06909-rif/nal was applied repetitively during the irrigation season in two citrus orchards over 3 years. In a mature (50-year-old) commercial citrus orchard covering 2.02 ha, weekly applications of *Pseudomonas putida* 06909-rif/nal with an in-field fermentor resulted in soil populations that fluctuated between 2.83 log CFU + 1 per g of soil and 4.35 log CFU + 1 per g of soil. Resulting rhizosphere populations of *Phytophthora parasitica* were significantly reduced in 1999 but not 1997 or 1998. In a newly planted citrus orchard, yearly applications of *Pseudomonas putida* 06909-rif/nal at the beginning of the irrigation season resulted in high soil populations of *Pseudomonas putida* 06909-rif/nal that

declined rapidly and never reduced the rhizosphere populations of *Phytophthora parasitica*. When *Pseudomonas putida* 06909-rif/nal was applied weekly, soil populations increased throughout the 1997 and 1998 irrigation seasons, reaching a maximum in 1998 and remained high throughout the 1999 irrigation season. Rhizosphere populations of *Phytophthora parasitica* were significantly reduced in 1998. Yearly applications of the fungicide metalaxyl and the nematicide phenamiphos reduced rhizosphere populations of *Phytophthora parasitica* in 1997 but not in 1998 or 1999. *Pseudomonas putida* 06909-rif/nal was uniformly distributed throughout the soil profile to a depth of 75 cm in both yearly and weekly applications. When applied through low-volume minisprinklers, *Pseudomonas putida* 06909-rif/nal was found in aerosols up to 3 m away.

Additional keywords: BioJect, frequent applications, inundative biological control.

Biological control has been proposed for the replacement of chemical control of plant diseases or for management of diseases that are not economically controlled with chemicals (2,3,10,13,18, 23,28). Despite the many advances made in understanding mechanisms of biological control, biocontrol agents for plant pathogens have achieved only limited commercial success. Failure of introduced biocontrol agents to establish in soil or on host roots has been associated with lack of disease control (6,12,22,26,27,31, 33,37).

Many researchers have suggested that insufficiencies in biological control may be overcome through inundative means (3), 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 over incorporation of bacteria-impregnated granules into the soil. Repetitive inoculations increased the nodulation effectiveness of a strain of *Rhizobium leguminosarum* (17). The frequency at which bacterial applications are made does not appear to affect the final soil population (30). By spreading the applications over time, the bacteria are present in the soil environment for a longer period and therefore available for biological control for a greater time period.

Eco Soil Systems (San Diego, CA) has developed a self-contained field fermentor, called the BioJect, to overcome the costs and difficulties inherent in repetitive applications of bioinoculants (Fig. 1). The BioJect is capable of producing 120 liters of bacterial

inoculum under field conditions. It has facilities to (i) sanitize water through sand and micron filtration followed by UV irradiation, (ii) heat and chill fermentations to maintain optimum temperatures, (iii) sanitize all equipment between successive fermentations with peracetic acid, and (iv) inject bacterial fermentations into an existing irrigation system, all through the use of an automated computerized controller. It can accommodate up to three concentrated media sources and three refrigerated inoculum sources, allowing flexible management strategies. It is currently registered with the Environmental Protection Agency as part of a commercial biocontrol system to control diseases of turf with a strain of *Pseudomonas aureofaciens*. This system was evaluated in a greenhouse environment by Steddom and Menge (30) and found to be effective.

Phytophthora root rot is an important disease of citrus, causing a slow decline that reduces both fruit size and number of fruit. Damage to young trees can severely stunt or kill them (15). The primary root rot pathogens in California are *Phytophthora parasitica* Dastur (= *Phytophthora nicotiana* Breda de Hann), which is active in the summer, and *Phytophthora citrophthora* (R.E. Sm. & E.H. Sm.) Leonian, which is active in the winter under Mediterranean climates (15). These pathogens can be found in nearly 100% of the citrus groves in California and Florida (20,32), causing an estimated \$76 million in loss to annual production in the United States (21). With their motile zoospores, both *Phytophthora* spp. can become epidemic with an exponential production of secondary inoculum (16).

There have been several studies concerning biological control of *Phytophthora* root rot of citrus (5,7-9,14,19,24,25,36). However, all of these biocontrol attempts were done in the laboratory or greenhouse and most of the attempts achieved only minimal success. Nemec et al. (22) tested several commercially available

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biocontrol agents inoculated onto roots of 7- to 9-month-old citrus seedlings before being transplanted to nursery fields. There are apparently no reports of biological control of *Phytophthora* root rot attempted under commercial field conditions. Turney (34) screened 1,600 rhizosphere bacterial isolates for suppression of *Phytophthora* root rot of citrus and found that 9.9% inhibited either *Phytophthora citrophthora* or *Phytophthora parasitica* in vitro. Of these 159 isolates, only 26% were effective at reducing rhizosphere populations of *Phytophthora citrophthora* or *Phytophthora parasitica* in the greenhouse. *Pseudomonas putida* 06909 was one of several isolates effective in seedling bed trials. This isolate was effective in reducing populations of *Phytophthora citrophthora* by 73 and 45% in two separate experiments in seedling beds. It was originally isolated as a contaminant on a colony of *Phytophthora citrophthora* recovered from the rhizosphere of citrus. This strain appears to be unique among bacterial biocontrol agents in that it actively colonizes the hyphae of *Phytophthora* spp. (38). Turney included *Pseudomonas putida* 06909 in his screen because of its remarkable colonization ability. It restricts the growth of *Phytophthora* spp. in vitro and produces an iron chelating siderophore, pyoverdine, but shows no evidence of antibiosis (35,38).

The objectives of this study were (i) to test the ability of *Pseudomonas putida* 06909-rif/nal to control *Phytophthora parasitica* root rot of citrus under field conditions; (ii) to test the efficacy of the Eco Soil Systems BioJect in growing and delivering *Pseudomonas putida* 06909-rif/nal under field conditions; and (iii) to determine if continuous applications of *Pseudomonas putida* 06909-rif/nal with every irrigation would increase the effectiveness of this strain against *Phytophthora parasitica* root rot of citrus.

MATERIALS AND METHODS

Field plots. Two field plots were established in California's Central Valley. The first was at the University of California Lindcove Research and Extension Center in Exeter, and the second at the Ferry Ranch in Woodlake. The Lindcove field plot was located 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 orange seedlings on troyer citrange rootstock were arranged in a randomized complete block design with 18 replicates, with one 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) weekly applications of *Pseudomonas putida* 06909-rif/nal with every irrigation, and (iv) an industry standard fungicide/nematicide treatment consisting of yearly spring applications of metalaxyl (Ridomil) at 2.35 liters/ha, and phenamiphos (Nemacur) 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 3-year 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 prior to irrigation. Two half-circle minisprinklers with

a flow rate of 20 liters/h were installed on each side of the base of each tree, spraying away from the trunk. 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. Bacteria were applied at the beginning of irrigation and the amount of bacteria applied at each irrigation was the same regardless of the length of irrigation. At the beginning and end of each irrigation season, irrigations were once every 14 days or as needed.

The Woodlake plot consisted of 50-year-old Valencia trees on sour orange rootstock. The soil was a San Joaquin sandy loam with the following characteristics: pH 7.7; electrical conductivity, 0.84 mmho/cm; organic matter, 0.88%; cation exchange capacity, 14.9 meq per 100 g; Ca, 3.5 meq/liter; Mg, 1.4 meq/liter; Na, 3.5 meq/liter; Cl, 2.4 meq/liter; B, 0.2 ppm; soluble Zn, 25.2 ppm; soluble Mn, 19.8 ppm; soluble Fe, 15.2 ppm; Cu, 7.7 ppm; N-total, 0.084%; and soluble K, 19.4 ppm. The grove was in a state of decline because of high populations of *Phytophthora parasitica*. Out of 111 trees, 34 similar trees were chosen for sampling. Seventeen were randomly assigned to either a water control treatment or a repetitive biocontrol treatment with *Pseudomonas putida* 06909-rif/nal. A prototype BioJect was modified to include a sanitation cycle and installed at the field site. This unit was used to produce the *Pseudomonas putida* 06909-rif/nal inoculum during the first irrigation season. A commercial BioJect was installed before the second irrigation season and used for the second and third years of the trial. The biocontrol agent *Pseudomonas putida* 06909-rif/nal was injected into the minisprinkler irrigation system, which serviced a 2.02-ha block of approximately 500 trees. The

Fig. 1. The EcoSoil Systems BioJect installed in a greenhouse at the University of California, Riverside.

irrigation line was split into two lines and the bacteria were injected into one line after the split, allowing uniform irrigation practices for all treatments. The second line delivered water without bacteria to the 17 control trees. The minisprinklers had a flow rate of 40 liters/h. Trees were irrigated once a week during most of the irrigation season, from approximately April to November, for 12 to 24 h as needed. The entire 25 liters of *Pseudomonas putida* 06909-rif/nal inoculum from the BioJect was injected into the irrigation lines in the first 4 h of irrigation regardless of the length of irrigation. At the beginning and end of each irrigation season, irrigations were once every 14 days, or as needed.

Growth of *Pseudomonas putida* 06909-rif/nal. *Pseudomonas putida* 06909-rif/nal (30) was used in all studies. This strain has been marked with antibiotic resistance to enable recovery from the soil. There was less than one organism per gram of soil from either field that could grow on mannitol glutamic acid yeast extract (MGY) rif/nal agar (30). *Pseudomonas putida* 06909-rif/nal was grown in both an in-field fermentor at Woodlake and in bottles for applications at Lindcove. The fermentor, an Eco Soil Systems BioJect, was controlled in a manner consistent with commercial installations of BioJects. The media used, MD1/2 (Eco Soil Systems), is a proprietary concentrated formulation consisting of glycerol, glutamic acid, proteose peptone, and phosphate buffer. The BioJect dilutes the growth medium at the initiation of a fermentation cycle with filtered, UV-irradiated potable water at a rate of 6 ml of concentrate per liter of water. Fermentations of 120 liters were carried out at either 25 or 35°C, with aeration for the entire cycle. *Pseudomonas putida* 06909-rif/nal grew well at both of these temperatures with an optimum at 30°C. All fermentations were for 12 h. Inoculum was prepared by streaking from a frozen stock culture onto MGY rif/nal. After 3 days, cells were scraped from the plate and suspended in 5 ml of sterile distilled water. This was then added to 1 liter of tryptic soy broth (Difco Laboratories, Detroit) in an un baffled 2-liter flask and shaken at 250 rpm on a rotary shaker at 24°C for 24 h. This stationary phase inoculum, with an average population of 1×10^9 CFU/ml, was then placed in sterile inoculum bags provided by Eco Soil Systems, and placed in the refrigerated compartment of the BioJect at 4°C. The BioJect was set to use 300 ml of inoculum per fermentation. Inoculum remained viable for 4 weeks, with a sharp decline after this time. Inoculum in the BioJect was changed at the

end of 3 weeks to maintain consistency. A sanitation cycle was automatically run after each fermentation cycle as designed by the manufacturer. The sanitation cycle rinses the fermentation vessel and lines with UV-irradiated water, and then with a concentrated solution of peracetic acid, and again with UV-irradiated water. Also 1/10 strength peracetic acid was used as a rinse for both the media and inoculum lines as recommended by Eco Soil Systems.

Hand application cultures also used MD1/2 at the same rates as described previously to maintain consistency between field sites. For growth at the Lindcove field station, a 5-liter nonbaffled polypropylene bottle was filled with 1.8 liters of sterile diluted media and a single 5-cm stir bar. A bottle was inoculated with approximately 10^6 CFU/ml of *Pseudomonas putida* 06909-rif/nal in the form of lyophilized inoculum in 10% skim milk 24 h prior to irrigation. The bottle was placed on a stir plate at room temperature. Bacterial growth in this system was slower than in the BioJect due to poor aeration, requiring twice as long to reach comparable numbers of bacteria.

Bulk soil populations of *Pseudomonas putida* 06909-rif/nal. Bacterial populations in bulk soil were monitored every 6 to 12 weeks by removing four soil cores (25×2.5 cm) equally spaced around each tree from the wetted area of the minisprinklers with an Oakfield tube. The top 5 cm of each core was discarded due to drying at the soil surface. The cores were pooled and mixed to provide a composite sample for each tree. Samples were transported to the lab in an insulated chest at ambient temperature. Within 24 h of sampling, soil was serially diluted, from 10^{-2} to 10^{-4} in distilled water (wt/vol), and spread on top of MGY rif/nal media. Plates were incubated at room temperature and counted after 3 to 4 days.

Rhizosphere populations of *Pseudomonas putida* 06909-rif/nal and *Phytophthora parasitica*. Rhizosphere populations of *Phytophthora parasitica* and *Pseudomonas putida* 06909-rif/nal were assessed in August 1997 and September 1998 and 1999 at both field sites. Rhizosphere populations of *Phytophthora parasitica* were assessed according to the method of Timmer et al. (32). This method is the best estimate of disease pressure for *Phytophthora* root rot of citrus. Rhizosphere soil that adhered to the citrus roots was collected from soil samples with a 5-cm bucket auger to a depth of 45 cm. Two cores were taken per tree at Woodlake, whereas only one core per tree was taken on the small trees at the

Fig. 2. Mean populations of *Pseudomonas putida* 06909-rif/nal in bulk soil at the Lindcove field site over 3 years. Treatments consist of a water control that received no bacteria, a yearly application of *Pseudomonas putida* 06909-rif/nal at the beginning of each irrigation season, or weekly applications of *Pseudomonas putida* 06909-rif/nal with every irrigation. Data were log-transformed before analysis of variance. Error bars derived from Waller-Duncan k-ratio *t* test.

Fig. 3. Mean populations of *Pseudomonas putida* 06909-rif/nal in bulk soil at the Woodlake field plot over 3 years. Treatments consist of water controls receiving no bacteria, or weekly applications of *Pseudomonas putida* 06909-rif/nal through the irrigation water using the EcoSoil Systems BioJect. Data were log-transformed before analysis of variance. Error bars represent Fisher's least significant difference ($\alpha = 0.05$).

Lindcove site. This soil was used to assess rhizosphere populations of *Phytophthora parasitica* and *Pseudomonas putida* 06909-rif/nal. *Phytophthora parasitica* was enumerated by placing a 1:10 (wt/vol) dilution of soil in distilled water in a petri dish with molten, cooled (50°C) PARPH agar (11) poured on top with mixing. Plates were incubated at room temperature, and colonies of *Phytophthora parasitica* were counted after 3 to 4 days. Enumeration of *Pseudomonas putida* 06909-rif/nal was the same as described for bulk soil enumerations.

Percolation of *Pseudomonas putida* 06909-rif/nal through soil. Bulk soil populations of *Pseudomonas putida* 06909-rif/nal at depths of 25, 50, and 75 cm were assessed on yearly application trees and weekly application trees at the Lindcove Field Station from four replicate blocks. The experiment was repeated on two successive weeks in September 1999. Samples from 25 cm deep were taken in the same manner as the bulk soil samples described previously. Samples from 50- and 75-cm depths were taken by extracting soil with a 5-cm bucket auger to the desired depth and aseptically taking a 10- × 2.5-cm core with a Oakfield tube that was scrubbed in water, sterilized with 95% ethanol, and dried between each sample. Care was taken to prevent contamination of the core, and only the bottom 5 cm of the core was kept for bacterial enumeration. Populations of *Pseudomonas putida* 06909-rif/nal were enumerated as described previously for bulk soil populations.

Movement through aerosols. Populations of *Pseudomonas putida* 06909-rif/nal moving through aerosol production during irrigation were monitored on weekly application treatments from three replicate blocks at the Lindcove Field Station. The experiment was repeated three times in July, August, and October 1999. Petri plates of MGY rif/nal agar, 100 × 2.5 mm², were opened and placed on the ground at distances of 1, 2, and 3 m in four directions around microsprinklers during the first hour of irrigation. After 1 h, the plates were covered and incubated at room temperature for 2 to 4 days before the number of bacterial colonies was recorded. The minisprinklers gave a wetted area radius of approximately 0.5 m. Plates that had more than 300 colonies were recorded as 300 to allow statistical analysis. The weather on all three dates was consistent, with temperatures between 17 and 20°C, 75 to 85% relative humidity, and with wind speeds below 1 m/s.

Statistics. Data were analyzed with analysis of variance in SAS (SAS Institute, Cary, NC). Variance in bacterial and fungal populations was stabilized through a log transformation, log₁₀(Y + 1), before analysis. Means were separated with the Waller-Duncan k-ratio *t* test for multiple comparisons and Fisher's least significant difference for pairwise comparisons. When data were combined over time, the analysis was run with a split plot by time model.

RESULTS

Bulk soil bacterial populations. When applied as single yearly applications, populations of *Pseudomonas putida* 06909-rif/nal in bulk soil at Lindcove were high initially with populations of

2.00 log CFU + 1 per g of soil in July 1997, 3.93 log CFU + 1 per g of soil in July 1998, and 3.70 log CFU + 1 per g of soil in July 1999, but quickly declined throughout the irrigation season (Fig. 2). When applied weekly through the irrigation system, the bulk soil populations steadily increased, reaching a maximum of 3.99 log CFU + 1 per g of soil in November 1997, 4.75 log CFU + 1 per g of soil in November 1998, and 4.50 log CFU + 1 per g of soil in September 1999, with only small declines over the winter. Mean populations of *Pseudomonas putida* 06909-rif/nal in the irrigation water at the Lindcove field site was 2 × 10⁶ CFU/ml, with a mean dilution rate of 1.5 × 10⁻³ for a 1-h irrigation. Between July and November, bacterial populations under the single yearly application treatment declined by a rate of 0.091, 0.165, and 0.112 log CFU/week for 1997, 1998, and 1999, respectively. In contrast, populations under the weekly application treatment increased by 0.091 and 0.069 log CFU/week for the same time period in 1997 and 1998, and declined by 0.018 log CFU/week in 1999. Between November and March, populations under weekly application trees declined by 0.045, 0.010, and 0.052 log CFU/week for 1997, 1998, and 1999, respectively. Populations of weekly applied bacteria were significantly higher than populations of yearly applied bacteria at all times, except dates directly following each yearly application (Fig. 2). Populations of the weekly applied bacteria appeared to level off after reaching a maximum population of 4.75 log CFU + 1 per g of soil in November 1998, with little change after that.

After weekly applications of *Pseudomonas putida* 06909-rif/nal, the bulk soil bacterial populations at the Woodlake plot were more variable than those at the Lindcove plot, with maximum populations of 3.49 log CFU/g of soil in July 1997, 3.87 log CFU/g of soil in September 1998, and 4.35 log CFU/g of soil in July 1999 (Fig. 3). Populations of *Pseudomonas putida* 06909-rif/nal in the irrigation water averaged 1.4 × 10⁷ CFU/ml of irrigation water during a 1-h irrigation. Soil populations of the weekly applied *Pseudomonas putida* 06909-rif/nal were always significantly higher than populations recovered from untreated trees. Again, there was a trend for continuously increasing populations when bacteria were applied repetitively.

Rhizosphere populations of *Pseudomonas putida* 06909-rif/nal and *Phytophthora parasitica*. *Phytophthora parasitica* populations at the Lindcove site were significantly reduced 92%

TABLE 2. Mean rhizosphere populations of *Pseudomonas putida* 06909-rif/nal at Lindcove in 1997 and 1999

Treatment	Rhizosphere populations of <i>Pseudomonas putida</i> 06909-rif/nal (log CFU + 1) ^y		
	1997	1999	1997-99 ^z
Water control	0.37 b	0.57 b	0.47 b
Yearly applications	0.74 b	3.33 a	1.30 b
Weekly applications	2.49 a	4.49 a	2.92 a

^y Means within the same column followed by the same letter are not significantly different according to the Waller-Duncan k-ratio *t* test (k-ratio = 100).

^z Data analyzed as a split plot by time model.

TABLE 1. Mean rhizosphere populations of *Phytophthora parasitica* at the Lindcove field plot from 1997 to 1999

Treatment	Rhizosphere populations of <i>Phytophthora parasitica</i> (lppg) ^y				
	1997	1998	1999	1998-99 ^z	1997-99 ^z
Water control	0.66 a	0.40 a	0.67 a	0.53 a	0.57 a
Yearly applications	0.58 a	0.45 a	0.60 a	0.53 a	0.55 a
Weekly applications	0.74 a	0.07 b	0.40 a	0.23 b	0.40 ab
Fungicide/nematicide	0.05 b	0.12 b	0.55 a	0.33 ab	0.24 b

^y Log propagules +1 per gram (lppg) of rhizosphere soil. Means within the same column followed by the same letter are not significantly different according to the Waller-Duncan k-ratio *t* test (k-ratio = 100).

^z Data analyzed as a split plot by time model.

TABLE 3. Mean populations of *Phytophthora parasitica* in the citrus rhizosphere at the Woodlake plot from 1997 to 1999

Treatment	Rhizosphere populations of <i>Phytophthora parasitica</i> (lppg) ^y			
	1997	1998	1999	1997-99 ^z
Water control	1.00 a	0.81 a	0.65 a	0.61 a
Weekly applications	0.98 a	0.60 a	0.65 a	0.47 a

^y Log propagules +1 per gram (lppg) of rhizosphere soil. Means followed by the same letters within rows are not significantly different according to Fisher's least significant difference ($\alpha = 0.05$).

^z Data analyzed as a split plot by time model.

in 1997 by the fungicide/nematicide treatment. In 1998, *Phytophthora parasitica* populations were reduced 70% by the fungicide/nematicide treatment and 84% by the weekly application biocontrol treatment when compared with the control (Table 1). Single yearly applications of *Pseudomonas putida* 06909-rif/nal never resulted in populations of *Phytophthora parasitica* different from water controls. When 1998 and 1999 were considered together, weekly applications of *Pseudomonas putida* 06909-rif/nal reduced *Phytophthora parasitica* by 57%. When 1997 through 1999 were combined, the fungicide/nematicide treatment reduced *Phytophthora parasitica* by 58%, and was not significantly different from the weekly applications of *Pseudomonas putida* 06909-rif/nal. In September 1997, rhizosphere populations of *Pseudomonas putida* 06909-rif/nal were significantly higher when applied weekly compared with when applied as a single yearly application in April 1997 (Table 2). In September 1999, rhizosphere populations of *Pseudomonas putida* 06909-rif/nal were not significantly different between yearly and weekly applied *Pseudomonas putida* 06909-rif/nal.

At Woodlake, *Phytophthora parasitica* populations were not significantly different between treatments until September 1999, when populations were reduced 80% by weekly applications of *Pseudomonas putida* 06909-rif/nal (Table 3). Rhizosphere populations of *Pseudomonas putida* 06909-rif/nal ranged from 5.2×10^3 CFU/g of soil in September 1999 to 1.46×10^4 CFU/g of soil in September 1998 at Woodlake. Rhizosphere populations of *Pseudomonas putida* 06909-rif/nal on trees receiving continuous applications of bacteria were always significantly higher than controls (Table 4).

Percolation of *Pseudomonas putida* 06909-rif/nal through soil. Populations up to 4.27 log CFU + 1 per g of soil were found 75 cm into the soil profile (Fig. 4). Populations were not different between depths from 25 to 75 cm, suggesting a uniform distribu-

TABLE 4. Mean populations of *Pseudomonas putida* 06909-rif/nal in the citrus rhizosphere at the Woodlake plot from 1997 to 1999

Treatment	Rhizosphere populations of <i>Pseudomonas putida</i> 06909-rif/nal (log CFU + 1 per g of soil) ^y			
	1997	1998	1999	1997-99 ^z
Water control	0.35 b	1.48 b	0.41 b	0.35 b
Weekly applications	4.02 a	4.17 a	3.72 a	3.73 a

^y Means followed by the same letters within rows are not significantly different according to Fisher's least significant difference ($\alpha = 0.05$).

^z Data analyzed as a split plot by time model.

Fig. 4. Mean populations of *Pseudomonas putida* 06909-rif/nal recovered at various soil depths from the Lindcove field plot. Bars with the same letters are not significantly different ($P > 0.05$).

tion throughout the soil profile. Weekly applications of *Pseudomonas putida* 06909-rif/nal resulted in higher populations than yearly applications, but even yearly applications had populations of 2.77 log CFU + 1 per g of soil. Samples were taken 18 and 19 weeks after the third yearly application. The presence of a hard pan precluded sampling at depths greater than 75 cm.

Movement through aerosols. During irrigation, detectable levels of *Pseudomonas putida* 06909-rif/nal could be found in aerosols up to 3 m from minisprinklers and up to 2.5 m from soil wetted by the minisprinklers (Fig. 5). Populations decreased with distance and were uniform in all directions around the sprinklers.

DISCUSSION

One of the biggest problems in biological control is maintaining a high population of an active biocontrol agent throughout the entire period of disease activity. Due to this, timing of applications of biocontrol agents is very important. Before the development of a fermentor suitable for use in a commercial field, studies concerning large areas of land or repetitive applications were difficult and cost prohibitive. To our knowledge, this is the first study to look at frequent applications of a biocontrol agent for control of a plant pathogen, and appears to be the first study looking at biological control of *Phytophthora* root rot in citrus trees older than 1 year.

Populations of *Pseudomonas putida* 06909-rif/nal remained high throughout the entire year when applied weekly through the irrigating season. When applied just once per year, populations of *Pseudomonas putida* 06909-rif/nal declined rapidly. This illustrates the problem with many biocontrol agents applied in single doses; they do not survive long enough at a sufficiently high level to provide biological control. The same results with single applications were seen in other studies (12,26). Our results concur with previous studies in which repetitive applications resulted in higher populations and better colonization (1,17). The high populations of *Pseudomonas putida* 06909-rif/nal found in the bulk soil resulted in high populations in the rhizosphere. The weekly applications resulted in more bacteria being applied than in the yearly application treatment. Steddom and Menge (30) showed that repetitive applications resulted in the same sized populations as a single application while maintaining soil populations over a longer time than a single application. Because *Phytophthora* root rot occurs over several months of the year, it is especially important to maintain high populations of a active biocontrol agent over time.

Populations of *Pseudomonas putida* 06909-rif/nal at Woodlake were not as large as those at Lindcove. Approximately 500 trees

Fig. 5. Mean number of bacterial colonies detected at distances from microsprinkler emitters at the Lindcove plot. Bars with the same letters are not significantly different according to Fisher's least significant difference ($\alpha = 0.05$).

were treated at Woodlake, and because the trees were 50 years old, the tree's roots occupy a larger volume of soil than those at Lindcove. The trees at Lindcove were 2-year-old nursery-grown trees, and only the experimental trees were treated. The Woodlake site was a commercial orchard and therefore it was subject to less stringent control of the biocontrol applications. The high levels of *Pseudomonas putida* 06909-rif/nal recovered from control trees could be attributed to commercial traffic and management practices; it is common practice to move irrigation lines during harvest to prevent damage to the sprinklers. Most of the background contamination could be attributed to a few sprinklers that had been misplaced and were subsequently moved back to their correct locations.

Although there was little biocontrol activity at either site, the results were encouraging for field trials of this magnitude. Biological control of *Phytophthora parasitica* at Lindcove was similar to chemical control, being different only in the first year. Because *Phytophthora parasitica* never reached an economically damaging level, there was little effect on yield, canopy volume, trunk diameter, root length, or tree ratings (29). The spent medium, which is injected into the irrigation lines with the bacteria, contains unutilized growth media, as well as metabolites of the bacteria. *Pseudomonas putida* 06909-rif/nal is not known to produce inhibitory compounds such as antibiotics, surfactants, or enzymes that would be effective against *Phytophthora parasitica*, but it does produce large quantities of the siderophore pyoverdine, evidenced by the green coloration of the growth media at the end of each fermentation. This system should be an effective way of delivering bacterial metabolites produced during growth. A slight increase in Fe³⁺ was seen in trees receiving bacterial applications, but the differences were rarely significant (29).

The BioJect proved to be an effective means of growing and delivering biocontrol agents. After installation of the commercial BioJect in the spring of 1998 at the Woodlake site, several fermentations aborted due to fermentation temperatures above the preset temperature of 24°C. The source of the problem was traced to excessively warm water entering the BioJect. The potable water that was used for the BioJect ran through a black polyethylene tube for approximately 500 m in partial shade with ambient temperatures of approximately 35°C. Fermentation temperatures were increased from 24 to 35°C to compensate, and the BioJect performed well after that. The initial temperature was chosen to match the average soil temperature. Misplaced emitters and missed fermentations resulted in more variable populations of *Pseudomonas putida* 06909-rif/nal at the Woodlake site compared with the Lindcove site. The BioJect was set for a 12-h fermentation, which allows two fermentations to be run in a single day. Using fermentation and dilution conditions identical to what we used in this experiment, a single BioJect could treat up to 70 acres a week. We have previously reported that 1:100,000 dilutions of *Pseudomonas putida* 06909-rif/nal were as effective as higher density inoculum in greenhouse studies (30). This would suggest it is possible that much larger acreages could be treated with a single BioJect.

The movement and distribution of *Pseudomonas putida* 06909-rif/nal through both aerosols and percolation through the soil profile is a source of concern. Although *Pseudomonas putida* 06909-rif/nal is considered a low risk organism, Cook et al. (4) defined hazards as risk plus exposure and pointed out that high exposure to low risk organisms is still a hazard. Practices that reduce exposure should be implemented before large populations of bacteria are released into the environment. It is also noted that this is not a danger inherent only in frequent applications, bacteria from yearly applications were also found in high numbers deep in the soil and would have been found in aerosols if applied through the irrigation system.

This study has demonstrated the utility of repetitive applications as a strategy for delivery of biocontrol agents to large, commer-

cial-scale fields. Frequent additions of low-density inoculum can effectively colonize soil, one of the major limitations of biological control of soilborne diseases. The in-field fermentor, the BioJect, is an effective way of growing and delivering a biocontrol bacterium to the roots of irrigated crops. Use of this technology has the potential to overcome inconsistencies due to a lack of survival or colonization seen in previous biocontrol studies.

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LITERATURE CITED

1. Bahme, J. B., Schroth, M. N., Van Gundy, S. D., Weinhold, A. R., and Tolentino, D. M. 1988. Effect of inocula delivery systems on rhizobacterial colonization of underground organs of potato. *Phytopathology* 78: 534-542.
2. Becker, J. O., and Schwinn, F. J. 1993. Control of soilborne pathogens with living bacteria and fungi: Status and outlook. *Pestic. Sci.* 37:355-363.
3. Cook, R. J. 1993. Making greater use of introduced microorganisms for biological control of plant pathogens. *Annu. Rev. Phytopathol.* 31:53-80.
4. Cook, R. J., Bruckart, W. L., Coulson, J. R., Goettel, M. S., Humber, R. A., Lumsden, R. D., Maddox, J. V., McManus, M. L., Moore, L. M., Meyer, S. F., Quimby, P. C., Stack, J. P., and Vaughn, J. L. 1996. Safety of microorganisms intended for pest and plant disease control: A framework for scientific evaluation. *Biol. Control* 7:333-351.
5. Daft, G. C., and Tsao, P. H. 1984. Parasitism of *Phytophthora cinnamomi* and *P. parasitica* spores by *Catenaria anguillulea* in a soil environment. *Trans. Br. Mycol. Soc.* 82:485-490.
6. Defago, G., Berling, C. H., Burger, U., Haas, D., Kahr, G., Keel, C., Voisard, C., Wirthner, P., and Wurthrich, B. 1990. Suppression of black root rot of tobacco and other root diseases by strains of *Pseudomonas fluorescens*: Potential applications and mechanisms. Pages 93-108 in: *Biological Control of Soil-Borne Plant Pathogens*. D. Hornby, ed. CAB International, Wallingford, UK.
7. El-Hamalawi, Z. A., Menge, J. A., and Pond, E. C. 1997. Effect of mulch and biocontrol agents on the control of *Phytophthora* diseases of citrus. (Abstr.) *Phytopathology* 87:S27.
8. Fang, J. G., and Tsao, P. H. 1995. Evaluation of *Pythium nunn* as a potential biocontrol agent against *Phytophthora* root rots of azalea and sweet orange. *Phytopathology* 85:29-36.
9. Fang, J. G., and Tsao, P. H. 1995. Efficacy of *Penicillium funiculosum* as a biological control agent against *Phytophthora* root rots of azalea and citrus. *Phytopathology* 85:871-878.
10. Harman, G. E., and Lumsden, R. D. 1990. Biological disease control. Pages 259-280 in: *The Rhizosphere*. J. M. Lynch, ed. John Wiley & Sons, West Sussex, England.
11. Kannwischer, M. E., and Mitchell, D. J. 1978. The influence of a fungicide on the epidemiology of blank shank of tobacco. *Phytopathology* 68:1760-1765.
12. Kim, D.-S., Weller, D. M., and Cook, R. J. 1997. Population dynamics of *Bacillus* sp. L324-92R-12 and *Pseudomonas fluorescens* 2-79RN-10 in the rhizosphere of wheat. *Phytopathology* 87:559-564.
13. Lewis, J. A., and Papavizas, G. C. 1991. Biocontrol of plant diseases: The approach for tomorrow. *Crop. Prot.* 10:95-104.
14. Lifshitz, R., Dupler, M., Elad, Y., and Baker, R. 1984. Hyphal interactions between a mycoparasite, *Pythium nunn*, and several soil fungi. *Can. J. Microbiol.* 30:1482-1487.
15. Lutz, A., and Menge, J. 1986. Citrus root health. II. *Phytophthora* root rot. *Citrograph* 72:33-39.
16. MacKenzie, D. R., Elliot, V. J., Kidney, B. A., King, E. D., Royer, M. H., and Theberge, R. L. 1983. Application of modern approaches to the study of the epidemiology of diseases caused by *Phytophthora*. Pages 303-313 in: *Phytophthora: Its Biology, Taxonomy, Ecology, and Pathology*. D. C. Erwin, S. Bartnicki-Garcia, and P. H. Tsao, eds. The American Phytopathological Society, St. Paul, MN.
17. Martensson, A. M. 1990. Competitiveness of inoculant strains of *Rhizobium leguminosarum* biovar *trifolii* in red clover using repeated inoculation and increased inoculum levels. *Can. J. Microbiol.* 36:136-139.
18. Mathre, D. E., Cook, R. J., and Callan, N. W. 1999. From discovery to use: Traversing the world of commercializing biocontrol agents for plant disease control. *Plant Dis.* 83:972-983.

19. May, L. L., and Kimati, H. 1999. Biological control of *Phytophthora parasitica* in citrus. Fitopatol. Bras. 24:18-24.
20. Menge, J. A., Johnson, E. L. V., Pond, E., Ferrin, D., Liu, H., Lutz, A., Strother, M., Bartnicki, D., Afek, U., and Sjoerdsma, J. 1988. Distribution and frequency of *Phytophthora parasitica* and *P. citrophthora* associated with root rot of citrus in California. (Abstr.) Phytopathology 78:1576.
21. Menge, J. A., and Nemec, S. 1997. Citrus. Pages 185-227 in: Soilborne Diseases of Tropical Crops. R. J. Hillocks and J. M. Waller, eds. CAB International, Wallingford, UK.
22. Nemec, S., Datnoff, L. E., and Strandberg, J. 1996. Efficacy of biocontrol agents in planting mixes to colonize plant roots and control root diseases of vegetables and citrus. Crop. Prot. 15:735-742.
23. Papavizas, G. C., and Lumsden, R. D. 1980. Biological control of soilborne fungal propagules. Annu. Rev. Phytopathol. 18:389-413.
24. Pemberton, C. M., Davey, R. A., Webster, J., Dick, M. W., and Clark, G. 1990. Infection of *Pythium* and *Phytophthora* species by *Olpidiopsis gracilis* (Oomycetes). Mycol. Res. 94:1081-1085.
25. Sawant, I. S., Sawant, S. D., and Nanaya, K. A. 1995. Biological control of *Phytophthora* root-rot of coorg mandarin (*Citrus reticulata*) by *Trichoderma* species grown on coffee waste. Indian J. Agric. Sci. 65:842-846.
26. Schippers, B., Bakker, A. W., and Bakker, P. A. H. M. 1987. Interactions of deleterious and beneficial rhizosphere microorganisms and the effect of cropping practices. Annu. Rev. Phytopathol. 25:339-358.
27. Schroth, M. N., and Becker, J. O. 1990. Concepts of ecological and physiological activities of rhizobacteria related to biological control and plant growth promotion. Pages 389-414 in: Biological Control of Soil-Borne Plant Pathogens. D. Hornby, ed. CAB International, Wallingford, UK.
28. Schroth, M. N., and Weinhold, A. R. 1986. Root-colonizing bacteria and plant health. Hortscience 21:1295-1298.
29. Steddom, K. 2000. The effects of continuous application of the biocontrol bacterium, *Pseudomonas putida* 06909-rif/nal, on *Phytophthora* root rot of citrus. Ph.D. diss. University of California, Riverside.
30. Steddom, K., and Menge, J. A. 2001. Evaluation of continuous application technology for delivery of the biocontrol agent *Pseudomonas putida* 06909-rif/nal. Plant Dis. 85:387-392.
31. Thomashow, L. S., and Weller, D. M. 1990. Application of fluorescent pseudomonads to control root diseases of wheat and some mechanisms of disease suppression. Pages 109-122 in: Biological Control of Soil-Borne Plant Pathogens. D. Hornby, ed. CAB International, Wallingford, UK.
32. Timmer, L. W., Menge, J. A., Pond, E., Miller, S. A., and Johnson, E. L. V. 1993. Comparison of ELISA techniques and standard isolation methods for *Phytophthora* detection in citrus orchards in Florida and California. Plant Dis. 77:791-796.
33. Troxler, J., Zala, M., Moenne-Loccoz, Y., Keel, C., and Defago, G. 1997. Predominance of nonculturable cells of the biocontrol strain *Pseudomonas fluorescens* CHA0 in the surface horizon of large outdoor lysimeters. Appl. Environ. Microbiol. 63:3776-3782.
34. Turney, J. 1995. The biological control of *Phytophthora* root rot of citrus using rhizobacteria. Ph.D. diss. University of California, Riverside.
35. Turney, J. K., Yang, C. H., Cooksey, D. A., and Menge, J. A. 1994. Mechanisms of biocontrol of *Phytophthora citrophthora* root rot of citrus by *Pseudomonas putida*. (Abstr.) Phytopathology 84:1091.
36. Weideman, H., and Wehner, F. C. 1993. Greenhouse evaluation of *Trichoderma harzianum* and *Fusarium oxysporum* for biological control of citrus root rot in soils naturally and artificially infested with *Phytophthora nicotianae*. Phytophylactica 25:101-105.
37. Weller, D. M. 1988. Biological control of soilborne plant pathogens in the rhizosphere with bacteria. Annu. Rev. Phytopathol. 26:379-408.
38. Yang, C. H., Menge, J. A., and Cooksey, D. A. 1994. Mutations affecting hyphal colonization and pyoverdine production in pseudomonads antagonistic toward *Phytophthora parasitica*. Appl. Environ. Microbiol. 60:473-481.