APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1997, p. 3776–3782 Vol. 63, No. 10
0099-2240/97/$04.00+0

Predominance of Nonculturable Cells of the Biocontrol Strain *Pseudomonas fluorescens* CHA0 in the Surface Horizon of Large Outdoor Lysimeters

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The persistence of the biocontrol agent *Pseudomonas fluorescens* CHA0 in the surface horizon of 12 large outdoor lysimeters planted with winter wheat, *Phacelia tanacetifolia* followed by spring wheat, or maize was monitored for 1 year. Soil was inoculated with a spontaneous rifampin-resistant mutant (CHA0-Rif) of CHA0, and the strain was studied by using colony counts, Kogure's direct viable counts, and total counts (immunofluorescence). The number of culturable cells of the inoculant decreased progressively from 8 to 2 log CFU/g of soil or lower. However, culturable cells of CHA0-Rif accounted for less than 1% of the total cells of the inoculant 8 months after release in autumn. Since viable but nonculturable cells represented less than a quarter of the latter, most cells of CHA0-Rif in soil were thus inactive-dormant or dead at that time. Nonculturable cells of the inoculant were predominant also in the surface horizon of the lysimeters inoculated in the spring, and a significant fraction of them were viable. Results suggest that the occurrence of nonculturable cells of CHA0-Rif was influenced by climatic factors (water availability and soil temperature) and the abundance of roots in soil. The fact that the inoculant persisted as mixed populations of cells of different physiological states, in which nonculturable cells were predominant, needs to be taken into account when assessing the autecology of wild-type or genetically modified pseudomonads released into the soil ecosystem.

The effective use of beneficial bacteria for protection of crops against soilborne pathogens requires the release of large numbers of wild-type or genetically modified cells into the soil environment, thus raising concerns about its ecological impact (9, 34). Information on persistence, dissemination, and physiological activity of biocontrol agents released into soil is an important aspect of risk assessment studies.

Most of our understanding of the autecology of pseudomonads in soil has been obtained by colony counts on selective media. These experiments have yielded considerable information on the influence of a wide range of environmental factors and strain properties on the behavior of pseudomonads in soil (7, 16, 22, 37, 38). However, colony counts tend to underestimate the actual number of bacteria in soil, as indicated by microscopic counts of cells after immunofluorescence (IF) staining (4, 5, 30). Soil microcosm experiments have shown that gram-negative bacteria, e.g., *Salmonella typhimurium* (36), a *Flavobacterium* sp. (11), *Alcaligenes eutrophus* (26), or *Pseudomonas fluorescens* (1), may lose their colony-forming ability once in soil, although Postma et al. (28) did not find any significant loss of culturability with *Rhizobium leguminosarum* introduced into nonsterile soil.

Several protocols have been proposed to assess whether cells unable to grow on routine culture medium are still viable. The one most frequently used is Kogure's direct viable count (DVC) (20), which is based on the assumption that viable cells can respond to the presence of nutrients (e.g., yeast extract) but do not necessarily form colonies on plates. The test is performed in the presence of nalidixic acid, which inhibits bacterial DNA synthesis (27) and interferes with cell division in many gram-negative bacteria. Therefore, viable cells enlarge without dividing and can be distinguished from nonviable cells, which remain small (20). Although it is not known with certainty whether all viable cells can respond positively to the treatment, since only one particular type of activity is studied, DVCs have been employed successfully to study the viability of bacteria in nonsterile soil (6, 11). The use of viability tests in combination with total cell counts and colony counts allows discrimination among culturable cells; viable but nonculturable (VBNC) cells, i.e., cells that display signs of physiological activity despite having lost the ability to grow on solid medium; and inactive-dormant cells, i.e., cells that do not respond to viability tests. In soil, the VBNC state has previously been observed with certain gram-negative bacteria (36) but was of marginal importance with others (11). A significant fraction of cells of *P. fluorescens* DF57 introduced into soil persisted in a VBNC state (1). However, this finding was derived from experiments performed with soil microcosms only, and whether they can be extended to the behavior of pseudomonads under field conditions is not known.

The objective of this study was to assess whether *P. fluorescens* CHA0-Rif persisted as culturable cells only or as mixed populations of culturable and nonculturable cells in the surface horizon of lysimeters grown with different crops. In addition to providing conditions similar to those in a field, lysimeters enabled comprehensive monitoring of climatic and soil water parameters. Inoculation was performed in autumn (lysimeters with winter wheat and those with *Phacelia tanacetifolia* followed by spring wheat) or spring (maize lysimeters). Population dynamics of CHA0-Rif in soil were studied by colony counts, DVCs, and total IF counts to monitor the physiological state(s) of the released pseudomonads.

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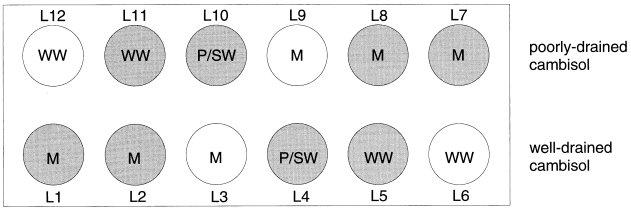

FIG. 1. Layout of the 12 lysimeters. Winter wheat (WW) was studied in lysimeters L5, L6, L11, and L12, *P. tanacetifolia* and spring wheat (P/SW) were studied in L4 and L10, and maize (M) was studied in L1 to L3 and L7 to L9. Lysimeters L1, L2, L4, L5, L7, L8, L10, and L11 were inoculated with CHA0-Rif (as indicated by a shaded background).

MATERIALS AND METHODS

Lysimeters. Twelve large outdoor lysimeters (200-cm diameter; 250-cm depth; about 8,000 kg of soil each) were used in the study (23). They were constructed in 1979 at the Eidgenössische Forschungsanstalt für Agrarökologie und Landbau (FAL; Zürich-Reckenholz, Switzerland), with the objective of investigating water transport and nutrient leaching in soils managed under standard crop rotations and contain either a well-drained cambisol (lysimeters L1 to L6) or a poorly drained cambisol (lysimeters L7 to L12 [Fig. 1]). The main differences between the two soils are the texture for horizons B_1 (sandy loam: clay, 15%; silt, 23%, for the well-drained cambisol; loam: clay, 16%; silt, 42%, for the other) and B_2 (sandy loam: clay, 17%; silt, 22%, for the well-drained cambisol; loam: clay, 26%; silt, 37%, for the other) and draining properties of the parental materials (23). The 30-cm-thick A_p horizons are essentially similar in size distribution of mineral particles (clay, 17% ± standard deviation [SD] of 2%; silt, 29% ± SD of 7%), texture (sandy loam to loam area of the textural triangle), profile of X-ray diffraction of clay fraction (14a), pH (7.4 ± SD of 0.1), organic matter content (2.9% ± SD of 0.4%), and soil structure (granular). Each lysimeter was set on a balance to measure daily fluctuations in weight and was equipped with a 10-cm-deep thermometer. The lysimeters had been under crop rotation for 14 years (essentially grass-based pasture, maize, rapeseed, and sugar beet), and the crops prior to the experiment were *Phacelia* (L1 to L3 and L7 to L9) or rapeseed (L4 to L6 and L10 to L12). Irrigation was not used.

Strain and inoculum preparation. The biocontrol agent *P. fluorescens* CHA0 (33) is effective against several soilborne fungal diseases of plants (16, 39). Experiments were carried out with the spontaneous rifampin-resistant mutant CHA0-Rif (22). The pseudomonad was grown overnight (with shaking) in liquid King's medium B (19) containing 100 μg of rifampin/ml (i.e., Rif100), and the cells were used to inoculate King's B agar. The plates were incubated overnight at 27°C, and the cells were harvested and suspended in sterile distilled water. The suspension was filtered through three layers of cheesecloth, and cell density was adjusted optically (600 nm) to 10 log CFU/ml (confirmed by colony counts on solid King's medium B containing Rif100).

Soil inoculation and crop systems. The current work was carried out from September 1993 to October 1994, and three crop systems were studied: winter wheat (L5 and L6 and L11 and L12), *P. tanacetifolia* (green manure) followed by spring wheat (L4 and L10), and maize (L1 to L3 and L7 to L9) (Fig. 1). Tillage was performed 1 day (spring wheat) or 1 week (*Phacelia*, winter wheat, and maize) prior to sowing. Inoculation of the surface of the lysimeters was performed by spraying 1 liter of cell suspension per m^2 (i.e., approximately 8 log CFU per g of 10-cm-thick surface soil), and the upper 10 cm of soil was then mixed with a spade. Inoculation was performed either on 20 October 1993 (winter wheat and *Phacelia*-spring wheat crop systems) or on 30 April 1994 (maize), as described below (Fig. 1).

Four of 12 lysimeters (L5, L6, L11, and L12) were sown with winter wheat cv. Galaxie (550 seeds/m^2) on 28 October 1993. The soil from two of them (L5 and L11) had been inoculated with CHA0-Rif 8 days prior to sowing, and the other two lysimeters (L6 and L12) were used as uninoculated controls. The plants were at the stage of seedlings (stage 16 to 19 [43]) when growth stopped in winter. The crop was harvested on 2 August 1994, and the corresponding lysimeters were left unplanted until the end of the experiment.

The lysimeters L4 and L10 were planted with *Phacelia* 5 weeks prior to inoculation with CHA0-Rif on 20 October 1993. The plants were well grown at that time, and a diligent effort was made to inoculate the soil uniformly. The shoots dried during winter and were incorporated into soil on 4 March 1994. Spring wheat cv. Frisal was sown immediately (550 seed/m^2). The crop was harvested on 2 August 1994, and the two lysimeters were left unplanted until the end of the experiment. Additional lysimeters cropped with *Phacelia* and spring wheat were not available, and so the study was performed without uninoculated control for this crop system.

The remaining six lysimeters were cropped with *Phacelia* during the autumn of 1993. Four of them (L1, L2, L7, and L8) were inoculated with CHA0-Rif on 29 April 1994, and maize (10 seeds/m^2) was sown on all four lysimeters 6 days later, as well as on lysimeters L3 and L9 (uninoculated controls). The crop was harvested on 30 October 1994.

Sampling, extraction, and dilution of soil. Soil samples (0.5 to 1 g each) were taken halfway between plants, at a depth of 5 to 10 cm from the surface. Until June 1994, roots were absent or rare in soil samples from the winter wheat and the maize lysimeters, indicating that bulk soil had been sampled. *Phacelia* was sown earlier than winter wheat and at a much higher seed density. Small roots were frequently observed in soil samples from the lysimeters growing *Phacelia*, which implies that these samples corresponded to a mixture of bulk soil and rhizosphere soil.

Four soil samples were collected at each lysimeter, and they were pooled for analysis. Each composite sample was transferred into a 100-ml Duran bottle containing sterile distilled water (final soil/water ratio of 1:10 [wt/vol]), and bottles were agitated for 60 min on a rotary shaker (350 rpm) and vortexed for 15 s. Dilution series were prepared in sterile distilled water.

Enumeration of bacterial cells. Extract and dilutions from each sample were spread plated onto S1 plates (10) containing Rif100, unamended S1 plates, and 10% tryptic soy agar (Difco) to recover the culturable cells of CHA0-Rif, the total culturable fluorescent pseudomonads, and the total culturable aerobic bacteria, respectively. Plates were incubated at 20°C for 6 days, and bacterial colonies were counted. No background resistance to rifampin was found when soil samples from uninoculated lysimeters were plated onto S1-Rif100 (detection limit about 10 CFU/g of soil). About 100 rifampin-resistant colonies (i.e., from the inoculated treatments) obtained in the last months of the experiment were checked by randomly amplified polymorphic DNA analysis with the primer D7 (17), and results confirmed that they corresponded to CHA0-Rif.

The DVC technique was used in combination with IF microscopy to quantify viable cells of CHA0-Rif. The soil extracts were diluted 50 times to reach a soil/water ratio of 1:500 (wt/vol). The resulting soil suspensions were incubated for 6 h in the presence of yeast extract (250 μg/ml) and nalidixic acid (20 μg/ml), in the dark and at room temperature, and were fixed with formaldehyde (final concentration, 20 mg/ml). Cell numbers of CHA0-Rif before and after the test were statistically identical, indicating that nalidixic acid successfully prevented population growth. After 30 s of vortexing, the samples (3 to 5 ml; 6 to 10 mg of soil) were immobilized by filtration onto polycarbonate filters (0.2-μm pore size, 25-mm diameter; Nuclepore; Costar, Cambridge, Mass.) and stained with Irgalan black (12). The filters were washed twice with 5 ml of 0.01 M phosphate-buffered saline (PBS; pH 7) containing 0.05% Tween 20 and 0.05% bovine serum albumin (i.e., washing buffer) and were treated with 1 ml of PBS containing 2% Tween 20 and 0.5% bovine serum albumin for 10 min. The primary antiserum used was specific for CHA0 (35), and no cross-reaction was found when soil samples from uninoculated lysimeters were studied. Nonspecific adsorption of the primary antibody to the soil background was reduced by a 3-min treatment of filters with 1 ml of a solution containing gelatin (2%) conjugated with rhodamine B-isothiocyanate (2). The filters were then washed three times with washing buffer and incubated for 60 min in the presence of the primary antiserum (diluted 1/1,000 in PBS). The filters were rinsed three times with 3 ml of washing buffer for 5 min. The secondary antibody consisted of fluorescein isothiocyanate-conjugated swine anti-rabbit immunoglobulins (Monosan SAR-3F; Am Uden, The Netherlands). The filters were incubated for 45 min in the presence of the secondary antibody (diluted 1/50 in PBS) and rinsed as described above. They were air dried and mounted with 1,4-diazobicyclo-(2,2,2)-octan-glycerol (DABCO-glycerol) mounting medium (13) to prevent fading. The filters were observed with a Zeiss Axioskop epifluorescence microscope (filters, 450 to 490 nm; magnification, ×400 and ×1,000). Cells of CHA0-Rif were counted over the entire filter (at least 20 fields and/or 200 bacterial cells). A total of two filters were studied for each sample. Elongated cells (4 to 10 μm in length) were considered viable cells, whereas small spherical cells (<1.5 μm in diameter) were counted as nonviable cells (i.e., inactive-dormant cells).

The total number of cells of CHA0-Rif was determined as the sum of viable cells and inactive-dormant cells of CHA0-Rif. Soil samples were also studied by IF microscopy without incubation in the presence of yeast extract and nalidixic acid, and total cell counts from the filters were statistically identical to those obtained when the DVC technique was used. The number of VBNC cells was obtained by subtracting colony counts from viable counts.

Statistical design and analyses. All cell counts were expressed per gram of soil. Before analysis, cell numbers and percentages were log and Arcsin transformed, respectively. Each combination of crop system (winter wheat, *Phacelia*-spring wheat, or maize) and inoculation regimen (inoculation with CHA0-Rif or no inoculation) was present in one well-drained cambisol and one poorly drained cambisol, except that only two lysimeters were available for the *Phacelia*-spring wheat system and both were inoculated (no uninoculated control). Analyses of variance performed with colony counts from the four inoculated maize lysimeters indicated that the type of cambisol (i.e., well-drained or poorly drained cambisol) had no significant influence on the results. Indeed, the levels of data fluctuation between two maize lysimeters with different cambisols were similar to those between two maize lysimeters with the same cambisol. Furthermore, the average SDs for the log of CFUs of CHA0-Rif were 0.35 (winter wheat), 0.14 (*Phacelia*-spring wheat), and 0.44 (maize) until August 1994 (see Fig. 3), indicating that the extent of data fluctuation within each treatment was modest. In conclusion, the type of cambisol did not influence results, and consequently, each lysimeter was considered as one replication, regardless of whether it contained a well-drained

or a poorly drained cambisol. For consistency, all results from the maize system with inoculation shown in this study were obtained with only two lysimeters (L1 and L2), as for the other treatments.

One composite soil sample from each lysimeter was investigated at each sampling time, which made two replications per treatment (i.e., each lysimeter considered as one replication). Analyses of variance (followed by Fisher's least significant difference tests when appropriate) were used to study differences at given sampling times or from one sampling time to the next ($P = 0.05$).

RESULTS

Total IF counts of *P. fluorescens* CHA0-Rif in soil. Strain CHA0-Rif was inoculated in October 1993 in the winter wheat and *Phacelia*-spring wheat lysimeters, and total counts of CHA0-Rif cells in soil were made until June 1994, after which cell density became too low for accurate counting by IF microscopy. In the surface horizon, the total number of CHA0-Rif cells had decreased from 8 to about 6 log CFU/g of soil by June 1994 in both crop systems (Fig. 2A and B). Strain CHA0-Rif was released at the end of April 1994 in the maize lysimeters, and the decline in the total number of cells of CHA0-Rif was faster in these lysimeters than in the others (Fig. 2C).

Colony counts of *P. fluorescens* CHA0-Rif in soil. In the winter wheat lysimeters, the number of culturable cells of CHA0-Rif in soil decreased from October 1993 to June 1994, and the decline in colony counts was more pronounced than that for total counts of the inoculant (Fig. 2A). Indeed, the culturable cells of CHA0-Rif represented about 90% of all cells of the strain at inoculation but only a small fraction of them later in the experiment (Table 1). Colony counts of the introduced pseudomonad were lower than 2 log CFU/g of soil 1 year after release. A similar trend was observed in the *Phacelia*-spring wheat lysimeters, but CFUs represented there a significantly higher percentage of all cells of CHA0-Rif than in the winter wheat lysimeters, at least during the first part of the experiment (Fig. 2B). In the maize lysimeters also, colony counts of CHA0-Rif declined over time (Fig. 2C). During the 2-month period following inoculation, culturable cells accounted for a significantly smaller percentage of the total cells of CHA0-Rif compared with results from the *Phacelia*-spring wheat lysimeters.

Kogure's DVCs of *P. fluorescens* CHA0-Rif in soil. Since most cells of CHA0-Rif became nonculturable in the surface horizon of the lysimeters, their physiological state was also monitored, by using the DVC method in combination with IF microscopy. Viable cells represented 100% of all cells of CHA0-Rif at inoculation (i.e., 8 log cells/g of soil). This percentage decreased over time, although the rates of decline for total cells and viable cells of CHA0-Rif were essentially similar during most of the experiment (Fig. 2). The number of viable cells of CHA0-Rif was about 6 log cells/g of soil in all inoculated lysimeters in June 1994. Whereas most viable cells of CHA0-Rif were able to form a colony on plates shortly after inoculation, the proportion of them still culturable declined to less than 2% in the winter wheat and *Phacelia*-spring wheat lysimeters at 202 days after inoculation and 13% in the maize lysimeters at 42 days after inoculation.

Effect of environmental factors on population dynamics of *P. fluorescens* CHA0-Rif in soil. Average daily values for water content and temperature of soil were recorded to evaluate whether there was any correlation between climatic events and population dynamics of the inoculant in the surface horizon, but no apparent relationship was found between the former and fluctuations in the numbers of total cells or viable cells of CHA0-Rif. For colony counts of the pseudomonad, however, the main drops (i.e., more than 1 log) in the winter wheat lysimeters, in November 1993 and February 1994, took place at

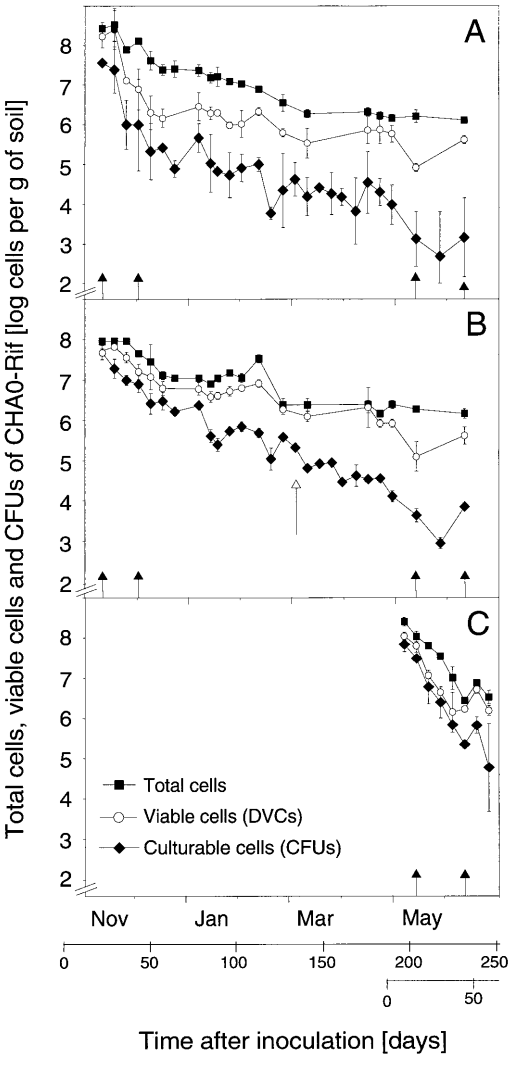

FIG. 2. Total cells, viable cells, and culturable cells of *P. fluorescens* CHA0-Rif in the surface horizon of lysimeters planted with winter wheat (A), *Phacelia*-spring wheat (B), or maize (C). The first bacterial release was on 20 October 1993. Winter wheat was sown 8 days after soil inoculation, and the crop was harvested on 2 August 1994. Inoculation in the *Phacelia* lysimeters was performed when the plants were 5 weeks old. On 4 March 1994, the dry shoots of *Phacelia* were incorporated into soil and spring wheat was sown (indicated with an open arrow in panel B). This crop was harvested on 2 August 1994. Maize was sown 6 days after the inoculation of soil on 29 April 1994. The crop was harvested on 30 October 1994. Values are mean cell densities ± SDs. Data used in Table 1 correspond to sampling times shown with solid arrows at the bottom of each panel.

two of four events when the average daily soil temperature fell to 0 to 1°C (Fig. 3B and C). Such values for average temperature imply that cells were subjected to subfreezing temperatures for parts of those days, i.e., that they could have undergone cycles of freezing-thawing. No such drop was observed in November in the lysimeters grown with *Phacelia*, and the corresponding decrease in February was modest.

Incorporation of dry *Phacelia* shoots into soil and subsequent sowing of spring wheat in March 1994 (indicated with an open arrow in Fig. 2B and 3C) did not coincide with any particular fluctuation in the numbers of total cells, or culturable cells of CHA0-Rif.

Comparison of colony counts for *P. fluorescens* CHA0-Rif, the total culturable fluorescent pseudomonads, and the total

TABLE 1. Persistence of cells of *P. fluorescens* CHA0-Rif in different physiological states in the surface horizon of lysimeters cropped with winter wheat, *Phacelia* followed by spring wheat (sown on 3 April 1994), and maize[a]

Crop system	Cell characteristic[b]	11 December 1993		12 March 1993		13 May 1994		6 October 1994	
		No. of cells[c]	% of total	No. of cells	% of total	No. of cells	% of total	No. of cells	% of total
Winter wheat	Culturable	7.55	16	5.99	2.0	3.13	0.14	3.16	0.16
	VBNC	8.11	49	6.81	6.7	4.91	6.5	5.62	17
	Inactive-dormant	7.94	35	8.07	91	6.07	93	6.02	83
	Total	8.42 (23 d)[d]	100	8.11 (44 d)	100	6.21 (202 d)	100	6.11 (230 d)	100
Phacelia-spring wheat	Culturable	7.69	56	6.89	18	3.64	0.24	3.85	0.21
	VBNC	7.15	16	7.15	32	5.35	15	5.46	23
	Inactive-dormant	7.32	28	7.33	50	6.16	85	6.04	77
	Total	7.96 (23 d)	100	7.66 (44 d)	100	6.27 (202 d)	100	6.16 (230 d)	100
Maize	Culturable					7.48	30	5.35	8.5
	VBNC					7.47	29	6.16	54
	Inactive-dormant					7.64	41	6.01	38
	Total					8.03 (14 d)	100	6.43 (42 d)	100

[a] The four sampling times chosen are indicated by solid arrows in Fig. 2.
[b] The cells of CHA0-Rif were either culturable, VBNC, or inactive-dormant. The numbers of VBNC cells were obtained by subtracting colony counts from viable counts, and those of inactive-dormant cells were obtained by subtracting viable counts from total counts.
[c] Log cells per gram of soil.
[d] Number of days (d) after inoculation.

culturable aerobic bacteria in soil. The numbers of total culturable fluorescent pseudomonads and total culturable aerobic bacteria in soil were monitored to evaluate whether the decrease in colony counts of CHA0-Rif paralleled quantitative changes at the level of these microbial communities. In the uninoculated lysimeters, the numbers of culturable fluorescent pseudomonads and culturable aerobic bacteria in the surface horizon were about 6 and 8 log CFU/g of soil throughout the 13-month experiment (Fig. 4).

Introduction of CHA0-Rif into soil in October 1993 in the winter wheat lysimeters (at 8 log CFU/g of soil) increased the total numbers of culturable fluorescent pseudomonads (by about 2 log CFU/g of soil [Fig. 4B]) and culturable aerobic bacteria (by less than 1 log CFU/g of soil [Fig. 4A]) for almost 1 month after inoculation. Although of shorter duration, a similar increase in the number of culturable fluorescent pseudomonads (but none for the culturable aerobic bacteria) was observed in May 1994 in the maize lysimeters, following inoculation with CHA0-Rif (Fig. 4C and D). CFUs of CHA0-Rif dropped throughout the experiment, and the percentage of the total culturable fluorescent pseudomonads represented by the culturable cells of CHA0-Rif decreased to 0.01% or less by October 1994 in all three crop systems.

DISCUSSION

This study was undertaken with the objective of evaluating, under outdoor conditions, whether colony counts were appropriate to monitor the persistence of the biocontrol agent *P. fluorescens* CHA0-Rif in soil. Results from total counts (IF microscopy), viable counts (Kogure's DVCs), and colony counts indicated that the inoculant was present in the surface horizon of all inoculated lysimeters as a mixed population of culturable cells, VBNC cells, and inactive-dormant cells (Table 1). Somewhat similar results have been obtained with another *Pseudomonas* inoculant in soil microcosms (1), but the current work strengthens those findings by extending observations to outdoor conditions essentially identical to those in a natural farm field.

Overall, the number of culturable cells of the inoculant in soil decreased over time in the current study, and decay rates for CFUs (Fig. 3), which are thought to depend on soil type and environmental conditions (22, 37, 40), were in the range of values reported for other *Pseudomonas* inoculants (37). Results showed that the decline in the numbers of total cells and (to a large extent) of viable cells of the inoculant over time was less pronounced than the reduction in its CFUs (Fig. 2). Thus the proportion of VBNC cells and inactive-dormant cells of CHA0-Rif increased over time as the predominance of culturable cells of the strain decreased, and the former physiological states were prevalent as early as 1 to 3 months after release, depending on the crop system (Fig. 2 and Table 1).

Colony counts indicated that for almost 2 months after inoculation, CHA0-Rif managed to remain at CFUs higher than the size of the resident culturable fluorescent *Pseudomonas* community normally sustained in this soil (Fig. 4). When expressed as a percentage of the total culturable fluorescent pseudomonads (data not shown), results showed that short-term variations in the CFUs of the inoculant presumably linked to climatic fluctuations or seasonal succession did not happen at the level of the resident culturable fluorescent pseudomonads, suggesting that this community had not been affected, at least from a quantitative point of view. This may be due in part to the fact that, in the short term, introduced bacteria tend to associate mainly with the dispersible clay fraction of soil, to which only a minority of the indigenous bacteria belong (29). However, different spatial locations of introduced and resident bacteria in the soil pore space cannot account entirely for the results obtained in the current work, over a 1-year period, as soil structuration is an ongoing process promoting the incorporation of microorganisms within soil aggregates.

Bacterial inoculants are challenged with a combination of nutrient starvation and environmental stresses in soil (37). Nutrient availability is generally low in soil outside the rhizosphere (42). Pseudomonads subjected to nutrient starvation in vitro underwent morphological (e.g., reduction of cell size) and physiological (e.g., lower level of metabolism) changes, became more resistant to abiotic stress (14, 21, 37, 38), and displayed a loss of culturability in some (21) but not all (14, 25) studies.

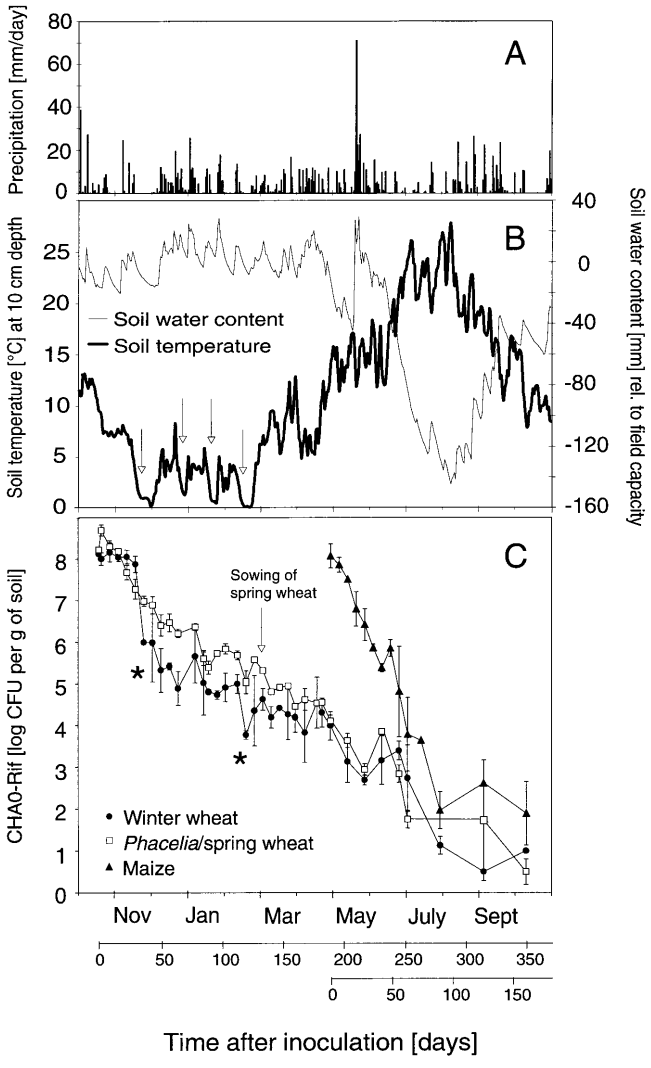

FIG. 3. Average agrometerological data during the experiment (values are means from all 12 lysimeters) and numbers of culturable cells of *P. fluorescens* CHA0-Rif in the surface horizon of the lysimeters. Total precipitation is indicated in millimeters per day (A). Daily average soil water content is expressed in millimeters of water relative to field capacity, and daily average soil temperature was measured at a depth of 10 cm (B). Means for colony counts of CHA0-Rif are shown ± SDs (C). In the winter wheat and the *Phacelia*-spring wheat lysimeters, the decay rates for CHA0-Rif were 0.29 to 0.37 and 0.20 log CFU/g of soil per 10 days for the first 3 and 8 months, respectively. In the maize lysimeters, the decay rate was 0.65 log CFU/g of soil per 10 days in the first 3 months after inoculation. Declines higher than 1 log CFU in the winter wheat lysimeters in November 1993 and February 1994 are shown with asterisks. They took place at two of four events (indicated with arrows in panel B) in which average daily soil temperatures reached 0 to 1°C.

This general response of starved cells is thought to enable their persistence in soil without compromising their ability to rapidly colonize roots subsequently growing in their vicinity (37). Indeed, introduction of *P. fluorescens* R2f Rp^r into soil microcosms led to loss of culturability and the development of a general resistance to abiotic stress similar to that observed under carbon starvation conditions in vitro (38). Therefore, nutrient limitation may be a reason why population dynamics of CHA0-Rif in soil were different in the winter wheat lysimeters (roots were almost never found in soil samples from them until June 1994) and the *Phacelia*-spring wheat lysimeters (small roots were present in soil samples from these lysimeters), which had been inoculated at the same time. Indeed, during the first 2 months after inoculation, the decline in the CFUs of CHA0-Rif was more steady and less pronounced in the *Phacelia*-spring wheat lysimeters than that in the winter wheat lysimeters (Fig. 3C), whereas total counts of the inoculant were statistically identical in both crop systems (Fig. 2A and B). For 3 months afterwards, colony counts of CHA0-Rif (and viable counts of the strain) remained significantly higher there than in the winter wheat lysimeters, but total counts of the pseudomonad were similar in both crop systems at all samplings but one.

A loss of culturability can be induced also by variations in temperature in *Pseudomonas* (25) and took place in *Bradyrhizobium japonicum* exposed to elevated temperature in soil (18). This suggests that the abiotic stresses identified in the current work may have contributed to the loss of colony-forming ability in CHA0-Rif (Fig. 3). Indeed, large reductions in the CFUs of the inoculant in the winter wheat lysimeters coincided with temperatures near the freezing point in winter (Fig. 3B). Loss of culturability for *A. eutrophus* and *Enterobacter cloacae* took place in air-dried soil microcosms but not when experiments were performed with moist soil (26). A similar phenomenon could have happened in the current work, from May to July 1994, i.e., when the lysimeters underwent the highest reductions in soil water content. In fact, this factor was probably more influential than the abiotic stresses taking place in late autumn-winter, as the 6-log decrease in the CFUs of CHA0-Rif in the lysimeters inoculated in October 1993 observed over an 8-month period took place within 3 months after inoculation only in the maize lysimeters (Fig. 3C). However, the decline in the number of total cells of the inoculant was also faster in the maize lysimeters than in the others.

Loss of culturability may be associated with different types of transformation in bacteria. First, cells having lost their ability to grow on plates may still be viable, and loss of culturability could have resulted then from physiological changes designed to increase their survival ability in the environment (31). Such VBNC cells have been detected on several occasions in *P. fluorescens* (14, 21, 25), including soil microcosm experiments (1). Second, cells could have died. Most heat-killed rhizobial cells disappeared within 2 weeks after their introduction into nonsterile soil (3), and similar results were obtained with dead cells of *B. japonicum* (8), *S. typhimurium* (36), and *Bacillus thuringiensis* (41). However, dead cells of CHA0-Rif were perhaps generated over time from viable cells of the inoculant, resulting in a constant supply of new dead cells. Third, nonculturable cells of CHA0-Rif that were not substrate responsive in the DVC test in the present study (Table 1) could have been dormant, a physiological state poorly understood for bacteria. Pedersen and Jacobsen (26) observed that cells of *E. cloacae* and *A. eutrophus* incubated in soil under drought stress did not respond to the DVC method, yet exhibited intact cell walls and DNA. It is thought that the majority of cells not displaying signs of viability but persisting in soil are unlikely to be dead (4).

Interestingly, VBNC cells may recover their former ability to form colonies on plates, and such resuscitation was achieved by a temperature upshift in *Vibrio vulnificus* (24) or the addition of nutrients in *Salmonella enteritidis* (32). Furthermore, the transformation of dormant cells of *Micrococcus luteus* into viable cells has been reported elsewhere (15). The VBNC and dormant states of pseudomonads introduced into the soil environment are poorly documented and understood, but their occurrence has important implications for the monitoring of wild-type or genetically modified *Pseudomonas* inoculants in a context of risk assessment. Further work in our laboratory will

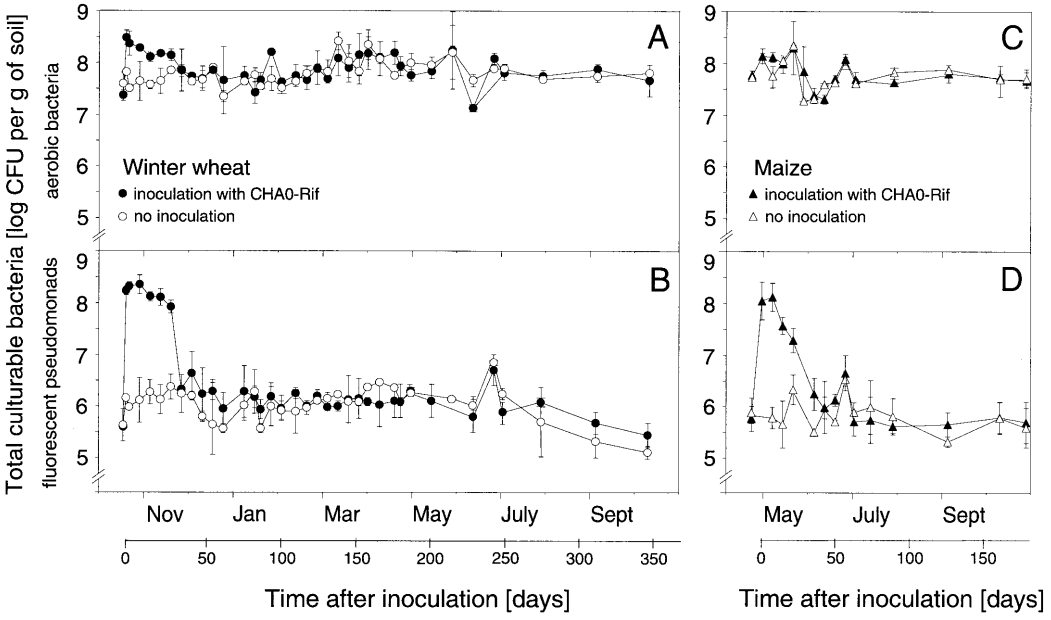

FIG. 4. Total culturable aerobic bacteria (A and C) and total culturable fluorescent pseudomonads (B and D) in the surface horizon of the winter wheat (A and B) and maize (C and D) lysimeters. The soil was inoculated with *P. fluorescens* CHA0-Rif or left uninoculated. Values are mean cell densities ± SDs.

target the ecological significance of those physiological states in the biocontrol agent *P. fluorescens* CHA0.

ACKNOWLEDGMENTS

This work was supported in part by the Swiss Federal Office for Education and Science (EU Biotech "IMPACT" project; PL 920053) and the Swiss National Foundation for Scientific Research (Priority Programme Biotechnology; projects 5002-035142 and 5002-04502311).

We are grateful to J. Nievergelt (FAL, Zürich-Reckenholz) and the staff at FAL for the opportunity to use the lysimeters, help with the experiments, and the data for rainfall, soil water content, and soil temperature. We thank C. Gyawali and A. Natsch from our laboratory for technical assistance and randomly amplified polymorphic DNA analysis of colonies, respectively.

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