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Autecology of the biocontrol strain *Pseudomonas fluorescens* CHA0 in the rhizosphere and inside roots at later stages of plant development

Joseph Troxler, Marcello Zala, Andreas Natsch, Yvan Moënne-Loccoz, Geneviève Défago *

Phytopathology Group, Institute of Plant Sciences, Swiss Federal Institute of Technology, CH-8092 Zürich, Switzerland

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Abstract

A spontaneous rifampicin-resistant mutant of the biocontrol agent *Pseudomonas fluorescens* CHA0 was released as soil inoculant in large outdoor lysimeters and its ability to colonise the roots of winter wheat, spring wheat (grown after *Phacelia*) and maize at the later stages of plant development was investigated by colony counts. The inoculant (i.e. CHA0-Rif) colonised the rhizosphere and the interior of the roots of both wheat varieties but CFUs at ripening were about 2 log (g root)$^{-1}$ or lower. In contrast, the roots of maize were colonised poorly by the pseudomonad at flowering, but the latter was found at 3 or more log CFU (g root)$^{-1}$ on and inside the roots in late ripening stage. Furthermore, CHA0-Rif was recovered at more than 5 log CFU (g root)$^{-1}$ from the interior of several maize root samples. Whereas most cells of CHA0-Rif in soil were small and did not respond to Kogure's viability test, the pseudomonad was present as viable, unusually large (7 mm long) rods inside maize roots. In a microcosm experiment performed with similar sandy-loam soil, the CFUs of maize root-associated CHA0-Rif were higher where the shoots of the plant had been cut off, confirming that older and/or decaying maize roots represent a favourable niche for the inoculant. Overall, the results indicate that *Pseudomonas* inoculants have the potential to colonise the roots of certain crops (e.g. maize but not wheat for strain CHA0-Rif) at later stages of plant development.

Keywords: *Pseudomonas*; Root colonisation; Culturability; Viability; Risk assessment

1. Introduction

Fluorescent pseudomonads have the potential to suppress soil-borne diseases and this beneficial effect may involve competition, antagonism and/or induced resistance mechanisms [1–4]. The ability of introduced pseudomonads to colonise the root at high population levels in competition with resident soil microorganisms is recognised as an important prerequisite for efficient biocontrol [1,5,6]. Therefore, pseudomonads have been modified genetically in an effort to develop better root colonisers [7,8].

Most studies on root colonisation by pseudomonads have been carried out over short time periods, since several biocontrol functions need to be exerted at the early stages of plant development. Indeed, the short-term dynamics of pseudomonads in the rhizo-

* Corresponding author. Tel: +41 (1) 632 38 69; fax +41 (1) 632 11 08; email: defago@ipw.agrl.ethz.ch

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sphere after inoculation is well documented. In natural soil microcosms, their CFUs in the rhizosphere can remain stable (or increase somewhat) for a short while before decreasing in time [8–12]. For instance, a spontaneous rifampicin-resistant mutant (i.e. CHA0-Rif) of the biocontrol agent *Pseudomonas fluorescens* CHA0 inoculated on wheat seedlings at 6.4 log CFU (root system)$^{-1}$ was recovered at 4.3 log CFU (root system)$^{-1}$ 21 days after planting in natural soil microcosms [13]. However, much less is known on the root-colonising ability of pseudomonads several months after inoculation.

Recently, Troxler et al. [14] have investigated the persistence of CHA0-Rif over a one-year period in the surface horizon of large outdoor lysimeters, with the objective to assess the population dynamics of the inoculant in soil in parallel with its vertical transport through a 2.5-m-thick soil profile. The results from colony counts showed that the introduced pseudomonad declined progressively to low CFUs in surface soil. However, the ecology of pseudomonads in the rhizosphere and in soil are different and it could be that the introduced strain persisted better in the vicinity of plant roots than in soil, as shown with other bacteria [15]. From a biosafety point of view, the possibility that bacteria associated with plant roots could persist in the environment well after their decline in bulk soil would have important implications, especially when dealing with genetically-modified strains [16,17]. The lysimeter experiment of Troxler et al. [14] provided an opportunity to determine comprehensively the persistence of *P. fluorescens* CHA0 in the soil environment, and to assess whether plant roots promoted long-term inoculant survival.

Therefore the objective of the current work was to investigate the autecology of the biocontrol agent *P. fluorescens* CHA0-Rif associated with the roots of winter wheat, spring wheat or maize in large outdoor lysimeters. A minisampling strategy was designed to obtain root samples for colony counts while minimising perturbation in the lysimeters. Early stages of plant development were deliberately ignored to focus on the persistence of the inoculant at later growth stages of plants. In soil, only a minority of cells of CHA0-Rif observed by immunofluorescence microscopy could be recovered by colony counts, and Kogure's Direct Viable Counts (DVCs; [18]) showed that a large fraction of the others were not substrate-responsive [14]. Thus immunofluorescence and the DVC method were also used in the current study, to investigate the ecology of the inoculant associated with the roots of maize.

2. Materials and methods

2.1. Inoculation of large outdoor lysimeters with *P. fluorescens* CHA0-Rif

The study made use of twelve large outdoor lysimeters (200 cm in diameter and 250 cm deep each) located at the FAL of Zürich-Reckenholz (Switzerland) and described in detail elsewhere [14,19]. Six of the lysimeters contain a well-drained cambisol and the other six a poorly-drained cambisol. The 30-cm-thick A_p horizon (sandy loam) was essentially the same in both types of cambisols.

The experiment was carried out from October 1993 to November 1994. Soil was inoculated with CHA0-Rif [13], a rifampicin-resistant mutant of *P. fluorescens* CHA0 [20], as follows. The strain was grown in liquid King's B medium [21] containing 100 μg of rifampicin ml^{-1} (i.e. Rif100). The culture was diluted in sterile distilled water and used to inoculate plates containing King's B agar (KBA). After incubation overnight, the cells were suspended in sterile distilled water. The suspension was filtered through three layers of cheese cloth and adjusted to a cell density of 10 log CFU ml^{-1}. The surface of the soil in the lysimeters was inoculated using one litre of cell suspension m^{-2} and the top 10 cm of soil was mixed with a spade (about 8 log CFU per g of 10-cm-thick surface soil).

2.2. Crop systems and experimental design

Crop systems (winter wheat, *Phacelia* followed by spring wheat, maize) and lysimeter management have been described previously [14]. Briefly, winter wheat cv. Galaxie was sown in four lysimeters previously cropped with rapeseed (550 seeds m^{-2}; October 28, 1993). Two of the lysimeters had been inoculated with CHA0-Rif on October 20, 1993 and the other two were left uninoculated. Two other lysimeters previously cropped with rapeseed were

sown with *Phacelia* (green manure) five weeks prior to inoculation with CHA0-Rif, again on October 20, 1993. The shoots dried during winter and were incorporated to soil prior to sowing of spring wheat cv. Frisal (550 seed m^{-2}) on March 4, 1994. The last six lysimeters were grown with *Phacelia* during the winter 1993/94. Four of these lysimeters were inoculated with CHA0-Rif on April 29, 1994 and maize cv. DK 200 (10 seeds m^{-2}) was sown on all six lysimeters on May 5, 1994. All four maize lysimeters inoculated with CHA0-Rif were used to assess whether persistence of the inoculant was similar in both types of cambisol (i.e. poorly-drained or well-drained cambisol), but results presented in Table 3 were obtained in only two of the four maize lysimeters inoculated with CHA0-Rif (i.e. one with a poorly-drained cambisol and one with a well-drained cambisol), as in the other two crop systems. Both wheat crops were harvested on August 2, 1994 and harvest of maize was carried out on October 30, 1994.

2.3. Sampling of roots

Root samples consisted of whole root systems (wheat seedlings; four samples per lysimeter) or small root segments (older wheat plants and maize; six samples per lysimeter). The root segments (one segment from each of six plants for a given lysimeter) were collected at a depth of 5-15 cm and were 4-10 cm in length. This minisampling strategy was chosen to minimise perturbation in the lysimeters. All root samples were shaken to remove loosely-adhering soil. Roots (and closely-adhering soil) were transferred each into a 100-ml Erlenmeyer containing 10 (wheat seedlings) or 20 ml (root segments) of sterile distilled water and rhizosphere soil extracts were obtained by shaking the bottles for 60 min at 350 rpm.

The roots were removed from the bottles and the presence of bacteria inside them was investigated after surface-sterilisation in 10% H_2O_2 for 15 s. The roots were then rinsed three times with sterile distilled water and ground aseptically in 10 ml of sterile distilled water using a mortar and a pestle. Root extracts were prepared by transferring the suspensions into 28-ml Universal bottles and shaking them for 30 min at 350 rpm. No colony arose on fluorescent pseudomonad-selective S1 plates [22] spread with aliquots from the last volume of water after rinsing of H_2O_2-treated root samples.

2.4. Enumeration and viability of bacterial cells

Dilution series of rhizosphere samples and root extracts were prepared in sterile distilled water. Culturable cells of CHA0-Rif were recovered on KBA amended with Rif100 and cycloheximide (190 μg ml^{-1}) and the total culturable fluorescent pseudomonads were enumerated on S1 plates. KBA-Rif100 was preferred to S1-Rif100 because it often gave higher counts (not with soil samples). No colony was found on plates containing Rif100 when samples from uninoculated lysimeters were studied.

Total and viable cells of the inoculant present inside the roots of maize were quantified using the DVC technique [18] and indirect immunofluorescence microscopy, as described by Troxler et al. [14]. Briefly, the extracts were diluted to reach a root/water ratio of 1:100 w/v. These suspensions were incubated in the presence of 0.025% yeast extract and 0.002% nalidixic acid for 6 h and then fixed with formaldehyde. The samples were fixed on polycarbonate membranes by filtration, treated with a polyclonal antiserum specific for CHA0 and stained with a FITC-labelled secondary antiserum that reacted with the primary antiserum. Observations were made using a epifluorescence microscope. No cross-reaction was found when studying samples from uninoculated maize lysimeters. The total number of FITC-stained cells was counted. Elongated FITC-stained cells (>10 μm) were determined as viable cells, whereas the others (i.e. not substrate-responsive) were counted as inactive/dormant cells.

2.5. Soil microcosm experiment

A soil microcosm experiment was carried out to further investigate the autecology of CHA0-Rif associated with the roots of maize. Sandy-loam soil similar to that in the A_p horizons of the lysimeters was collected from Eschikon soil [13], sieved (5 mm) and inoculated by spraying a cell suspension of CHA0-Rif (4 log CFU per g soil). The soil was mixed thoroughly and used to fill 30 pots (250 g soil per pot; bulk soil density of about 1.5). Seeds of maize cv.

Table 1
Colonisation [a] of the roots of winter wheat by *P. fluorescens* CHA0-Rif and resident culturable fluorescent pseudomonads

Date sampling and number of days after inoculation [b]	Growth stage of wheat [c]	Total culturable fluorescent pseudomonads (log CFU per g fresh root) [d]				Culturable cells of CHA0-Rif [e]					
		Rhizosphere		Interior root		log CFU (g fresh root)$^{-1}$		% of the root samples colonised		% of the total culturable fluorescent pseudomonads	
		Control	Inoculated	Control	Inoculated	Rhizosphere	Interior root	Rhizosphere	Interior root	Rhizosphere	Interior root
17.12.93 (58 d)	Seedling	6.3 ± 0.4 a	6.1 ± 0.5 a	5.1 ± 0.3 b	4.8 ± 0.3 b	5.5 ± 0.4 a	4.8 ± 0.3 b	100 a	100 a	12 a	80 b
09.05.94 (197 d)	Flowering	6.2 ± 0.6 a	5.9 ± 0.4 a	5.3 ± 0.7 b	5.1 ± 0.5 b	3.9 ± 0.4 a	3.5 ± 0.7 a	100 a	100 a	0.89 a	2.5 a
27.07.94 (276 d)	Ripening	5.0 ± 0.3 a	5.5 ± 0.6 a	5.0 ± 0.7 a	5.2 ± 0.5 a	0.76 ± 1.8 a (3.0 ± 0.4)	2.1 ± 0.2 a (2.4 ± 0.3)	25 a	75 b	0.0018 a (0.32)	0.079 a (0.16)

[a] All CFUs are means ± standard deviation.

[b] Inoculation of soil with CHA0-Rif was performed eight days before sowing. Harvest was carried out six days after the last sampling.

[c] Seedling, flowering and ripening corresponded to growth stages 16-19, 60-63 and 95, respectively [24].

[d] Within each sampling time, the statistical relationships between the four inoculation x root zone combinations are indicated with the letters a and b. In soil, the numbers of total culturable fluorescent pseudomonads were 6.3 (17.12.93), 6.1 (09.05.94) and 6.1 log CFU (g soil)$^{-1}$ (27.07.94).

[e] The statistical relationships between rhizosphere and the interior of roots within sampling times are shown with letters a and b. The detection limit was 1.8 log CFU (g fresh root)$^{-1}$. Results in brackets were obtained using only the samples in which CHA0-Rif was detected. In soil, the numbers of culturable cells of CHA0-Rif (on S1+Rif100) were 5.4 (17.12.93), 3.1 (09.05.94) and 1.1 log CFU (g soil)$^{-1}$ (27.07.94), and they represented 1.3% (17.12.93) and 0.14% (09.05.94) of the total number of culturable fluorescent pseudomonads.

Table 2
Colonisation [a] of the roots of spring wheat by *P. fluorescens* CHA0-Rif and resident culturable fluorescent pseudomonads

Date sampling and number of days after inoculation [b]	Growth stage of wheat [c]	Total culturable fluorescent pseudomonads in lysimeters inoculated with CHA0-Rif (log CFU per g fresh root) [d]		Culturable cells of CHA0-Rif [e]					
				log CFU (g fresh root)$^{-1}$		% of the root samples colonised		% of the total culturable fluorescent pseudomonads	
		Rhizosphere	Interior root	Rhizosphere	Interior root	Rhizosphere	Interior root	Rhizosphere	Interior root
09.05.94 (197 d)	Flowering	5.8 ± 0.4 a	5.4 ± 0.7 b	3.9 ± 0.4 a	3.7 ± 0.8 a	100 a	100 a	1.3 a	2.0 a
27.07.94 (276 d)	Ripening	5.3 ± 0.3 a	4.9 ± 0.3 b	0.4 ± 0.8 a (1.6 ± 0.1)	2.1 ± 1.6 a (2.8 ± 1.0)	25 a	75 b	0.0012 a (0.020)	0.14 a (0.79)

[a] All CFUs are means ± standard deviation. Within each sampling time, the statistical relationships between rhizosphere and the interior of roots are shown with letters a and b.
[b] Inoculation of soil with CHA0-Rif (20.10.93) was performed in lysimeters with a five-week-old stand of *Phacelia*. Spring wheat was sown on 04.03.94, i.e. 134 days after soil inoculation. Harvest was carried out six days after the last sampling.
[c] Flowering and ripening corresponded to growth stages 60-63 and 95, respectively [24].
[d] In soil, the numbers of total culturable fluorescent pseudomonads were 6.2 (09.05.94) and 5.7 log CFU (g soil)$^{-1}$ (27.07.94).
[e] The detection limit was 1.8 log CFU (g fresh root)$^{-1}$. Results in brackets were obtained using only the samples in which CHA0-Rif was detected. In soil, the numbers of culturable cells of CHA0-Rif (on S1+Rif100) were 3.6 (09.05.94) and 1.8 log CFU (g soil)$^{-1}$ (27.07.94), and they represented 0.25% of the total number of culturable fluorescent pseudomonads on 09.05.94.

Silex 170 were surface-disinfected and germinated on 0.6% water agar [23]. Three-day-old seedlings were transferred to pots (one seedling per pot) immediately after inoculation and the pots were put in a growth chamber (70% relative humidity) with 16 h of light (160 μE m^{-2} s^{-1}; 22°C) and 8 h of dark (18°C). The water content of the soil was adjusted to 20% w/w (i.e. water potential of about -0.03 MPa) with distilled water.

At 20 days after inoculation, the shoots were cut off in 10 of 20 pots and the experiment was continued for another 20 days, with the objective of comparing the influence of healthy and dying roots on the population dynamics of the inoculant and the resident fluorescent pseudomonads. Root colonisation was assessed by colony counts at 20 days (10 pots) and 40 days after inoculation (10 pots for each of the two treatments). Plants were processed individually. Whole root systems were washed briefly by gentle shaking in sterile distilled water (10 s) and were ground aseptically using a mortar and pestle. Extraction, dilution and plating of samples were performed as in the lysimeter experiment.

2.6. Design and statistical analyses

The current work was carried out using twelve lysimeters. Each combination of crop system and inoculation treatment studied was present in two lysimeters, one containing a poorly-drained cambisol and the other a well-drained cambisol. All root samples were taken from the A_p horizon, which was essentially the same in both types of cambisol and therefore both types of cambisols were considered as being identical in the study. In fact, the type of cambisol had no effect on the dynamics of culturable cells of CHA0-Rif in surface soil, as shown when comparing the CFUs of the inoculant in two lysimeters containing a poorly-drained cambisol and two lysimeters containing a well-drained cambisol [14]. Likewise, data from the same lysimeters indicated that the type of cambisol had no influence on root colonisation in the current work (data not shown).

All statistical analyses were performed at $P = 0.05$ level. CFUs and percentages were log-transformed and Arcsin-transformed, respectively. The detection limit for colony counts was 60 (i.e. 1.8 log) CFU (g

Table 3
Colonisation [a] of the roots of maize by *P. fluorescens* CHA0-Rif and resident culturable fluorescent pseudomonads

Date sampling and number of days after inoculation [b]	Growth stage of maize [c]	Total culturable fluorescent pseudomonads (log CFU per g fresh root) [d,e]				Culturable cells of CHA0-Rif					
						log CFU (g fresh root)$^{-1}$ [d,f]		% of the root samples colonised [d]		% of the total culturable fluorescent pseudomonads [d]	
		Rhizosphere		Interior root							
		Control	Inoculated	Control	Inoculated	Rhizosphere	Interior root	Rhizosphere	Interior root	Rhizosphere	Interior root
29.07.94 (91 d)	Flowering	4.6 ± 0.6 a	4.9 ± 0.6 a	< 1.8	1.5 ± 1.4 (3.6 ± 0.5)	0.9 ± 1.3 (2.3 ± 0.6)	< 1.8	35 a	0 b	0.011 (0.25)	Not applicable
21.10.94 (175 d)	Ripening	5.7 ± 0.2 a	5.8 ± 0.7 a	5.3 ± 1.0 a	5.6 ± 0.4 a	0.9 ± 1.5 a (3.0 ± 1.0 a)	1.7 ± 2.2 a (4.1 ± 1.2 b)	40 a	40 a	0.0013 a (0.16 a)	0.012 a (3.2 b)
25.10.94 (179 d)	Ripening	6.6 ± 0.4 a	6.6 ± 0.6 a	5.8 ± 0.5 b	6.8 ± 0.6 a	2.8 ± 1.8 a (3.8 ± 0.9 a)	3.6 ± 2.2 a (4.9 ± 0.6 b)	75 a	75 a	0.016 a (0.16 a)	0.063 a (1.3 b)
01.11.94 (186 d)	Harvest	Not done	6.2 ± 0.5 a	Not done	7.0 ± 0.5 b	2.9 ± 1.8 a (3.9 ± 0.9 a)	3.6 ± 2.2 a (5.0 ± 0.6 b)	75 a	75 a	0.050 a (0.50 a)	0.040 a (1.0 a)

[a] CFUs are means ± standard deviation. The detection limit was 1.8 log CFU (g fresh root)$^{-1}$. Results in brackets were obtained using only the samples that yielded colonies.
[b] Inoculation of soil with CHA0-Rif was performed six days before sowing. The last samples (01.11.1994) were taken two days after harvest.
[c] Flowering and ripening corresponded to stages 8 and 9.5, respectively, in the USDA system.
[d] Statistical relationships within each sampling time are indicated with the letters a and b.
[e] In soil, the numbers of total culturable fluorescent pseudomonads were 5.6 (29.07.94), 5.7 (21.10.94) and 5.7 log CFU (g soil)$^{-1}$ (01.11.94).
[f] In soil, the numbers of culturable cells of CHA0-Rif (on S1+Rif100) were 2.4 (29.07.94), 1.8 (21.10.94) and 1.2 log CFU (g soil)$^{-1}$ (01.11.94).

fresh root)$^{-1}$ and an arbitrary log value of zero was chosen when no colony was found on the plates. In the lysimeter experiment, two-factor analyses of variance (lysimeter considered as one factor) were performed to identify differences at given sampling times (e.g. uninoculated control vs. inoculation with CHA0-Rif; rhizosphere vs. interior root; winter wheat vs. spring wheat) or from one sampling time to the next within given crop systems.

A randomised design was used for the soil microcosm experiment. The results obtained 40 days after inoculation were studied by analysis of variance to compare the two treatments.

3. Results

3.1. Colonisation of the rhizosphere and of the interior of roots by P. fluorescens strain CHA0-Rif

In October 1993, *P. fluorescens* strain CHA0-Rif was added to the soil of lysimeters that were subsequently (eight days later) sown with winter wheat. The inoculant colonised both the rhizosphere and the interior of the roots, but colony counts showed that its population size declined from the seedling to the flowering stages (Table 1). However, CHA0-Rif could still be recovered from the rhizosphere and the interior of roots at the ripening stage (i.e. 276 days after inoculation). Whereas the inoculant was found in a majority of interior root samples then, it was detected in only 25% of all rhizosphere samples (Table 1).

Lysimeters grown with a five-week-old stand of *Phacelia* were also inoculated with CHA0-Rif in October 1993, and colonisation of the roots of subsequent spring wheat (sown in March 1994) was investigated by colony counts. Again, the inoculant was recovered from both the rhizosphere and the interior of roots, and its CFUs decreased from flowering to ripening (Table 2). As for winter wheat, CHA0-Rif was found more often in the interior of root samples than in the rhizosphere of spring wheat at ripening.

The maize lysimeters were inoculated with CHA0-Rif in April 1994, six days prior to sowing of maize. In contrast to the roots of winter wheat and spring wheat, those of maize were not colonised (interior root) or infrequently colonised and at low CFUs (rhizosphere) by the inoculant at flowering (Table 3). However, CHA0-Rif was recovered more frequently and at higher levels from the rhizosphere and the interior of roots at the last two samplings (i.e. at ripening and two days after harvest). For instance, the population of the inoculant was higher than 5 log CFU (g fresh root)$^{-1}$ in the interior of several root samples at the last sampling.

In conclusion, CHA0-Rif colonised the rhizosphere and the interior of the roots of all three plants studied. Whereas CFUs of the inoculant associated with the roots of winter wheat and spring wheat decreased in time, they increased at the later stages of plant development in the case of maize.

3.2. Total number of culturable fluorescent pseudomonads and impact of P. fluorescens CHA0-Rif

The inoculation with CHA0-Rif had essentially no

Table 4
Morphology of cells of *P. fluorescens* CHA0-Rif in liquid culture, in soil and inside the roots of maize

Location of CHA0-Rif	Cell length (μm) [a]	Occurrence of flagella
Nutrient-yeast extract broth [b]	3.6 ± 1.1 a	1-4 flagella
Soil [c]	1.5 ± 0.4 b	Few cells with flagella
Inside the roots of maize [d]	6.8 ± 1.3 c	1-4 flagella

[a] A total of 50 cells were studied in each case. All values are means ± standard deviation. Data were studied by analysis of variance followed by Student *t* tests ($P = 0.05$). Statistical differences are indicated with letters a, b and c.
[b] Overnight culture containing 9 log CFU ml^{-1}.
[c] The soil was sampled from the surface horizon of lysimeters cropped with maize, on 24.06.94 (56 days after inoculation). The numbers of total cells, viable cells and culturable cells of CHA0-Rif were 6.6, 6.3 and 5.0 log (g soil)$^{-1}$, respectively.
[d] The roots were sampled on 25.10.94 (i.e. 179 days after soil inoculation, 173 days after sowing and 5 days before harvest; ripening stage). Most cells of CHA0-Rif inside the roots had a length between 6 and 7.5 μm (min. 4.5 μm; max. 8.5 μm).

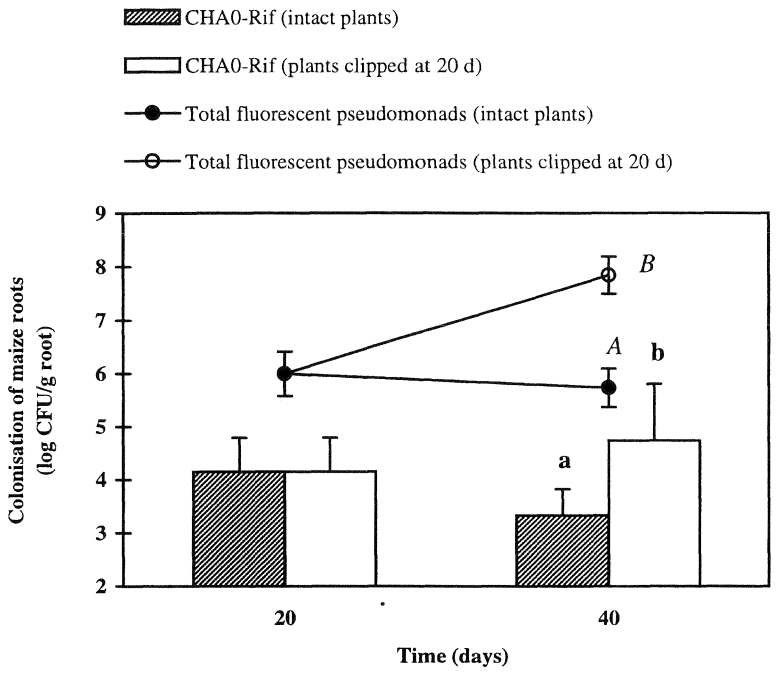

Fig. 1. Colonisation of the root system of maize by *P. fluorescens* CHA0-Rif introduced as soil inoculant (4 log CFU g^{-1}). Error bars represent standard deviations. At 40 days after inoculation, the numbers of culturable cells of CHA0-Rif and of the total fluorescent pseudomonad associated with maize roots were statistically higher in microcosms where the shoots had been cut off 20 days earlier, as indicated by the letters **a**,**b** and *A*,*B*, respectively.

effect on the total number of culturable fluorescent pseudomonads in the rhizosphere or the interior of the roots of winter wheat (Table 1) and maize (Table 3) at the growth stages studied. For winter wheat (Table 1) and spring wheat (Table 2), the size of the culturable population of all fluorescent pseudomonads in the rhizosphere and in the interior of roots was generally similar from one sampling to the next, and was also similar for both plants at each sampling time. In contrast, the total number of culturable fluorescent pseudomonads associated with the roots of maize increased from flowering to ripening, especially in the interior of the roots, which were little colonised at flowering (Table 3).

When expressed as a percentage of the total culturable fluorescent pseudomonads, the number of culturable cells of CHA0-Rif decreased in time in the rhizosphere and in the interior of the roots of winter and spring wheats (Tables 1 and 2). This decline was not observed in the case of maize whose roots were colonised by CHA0-Rif (Table 3).

3.3. *Morphology and viability of P. fluorescens strain CHA0-Rif inside the roots of maize*

Immunofluorescence microscopy was used to investigate the morphology of cells of CHA0-Rif in soil from the surface horizon of the maize lysimeters and inside the roots of maize at ripening. Winter and spring wheats were not studied because cell numbers were too low for accurate determinations by immunofluorescence. The pseudomonad was found mostly as small spherical cells (usually without flagella) in soil and as 7-μm long rods (with flagella) inside the roots of maize (Table 4). The latter cells were almost twice as long as those of CHA0-Rif in an overnight nutrient-yeast extract liquid culture.

At ripening, the total number of cells of CHA0-Rif inside the roots of maize was 5.7 log (g fresh root)$^{-1}$, whereas CFUs amounted to 4.9 log CFU (g fresh root)$^{-1}$ only. Kogure's DVCs showed that all cells of CHA0-Rif were viable inside the roots of maize. In conclusion, CHA0-Rif was found as large rod-shaped viable cells in the interior of the roots of

maize and 26% of them could be recovered by colony counts.

3.4. Colonisation of the root system of maize in soil microcosms

Since CHA0-Rif and the resident fluorescent pseudomonads were found to extensively colonise the rhizosphere and the interior of the roots of maize at later stages of plant development (e.g. during ripening), a soil microcosm experiment was carried out to evaluate whether these phenomena could be reproduced under semi-controlled conditions. Decaying roots were obtained in situ artificially, by cutting off the shoots of maize 20 days after the transfer of aseptically-grown seedlings into soil. Indeed, 20 days after cutting, the roots of plants without shoots displayed signs of mechanical weakness, whereas the roots of intact maize were healthy. Colony counts showed that the culturable population of the inoculant associated with live maize roots decreased by almost one log from day 20 to day 40 after inoculation (Fig. 1). A decline (from 5.8 to 4.4 log CFU per g root) was also observed from days 20 to 40 when CHA0-Rif had been inoculated onto seedlings instead of in soil (data not shown). At 40 days after inoculation, both the inoculant and the total fluorescent pseudomonads were recovered at higher CFUs from the roots of plants whose shoots had been cut off than from those of intact maize, regardless of whether CHA0-Rif had been added to soil (Fig. 1) or to the seedlings (data not shown).

4. Discussion

So far, most information on the ability of the biocontrol agent *P. fluorescens* CHA0-Rif to colonise roots has been obtained in soil microcosm experiments not exceeding two months [13,25]. In the current work, the pseudomonad was introduced as a soil inoculant into the surface horizon of large outdoor lysimeters and the role of roots in its persistence was studied at the later stages of plant development.

Colony counts showed that the culturable population of CHA0-Rif (inoculated in October 1993) in the rhizosphere of winter wheat decreased in time (Table 1), as observed usually with other pseudomonads released in the field [26,27]. In the current work, the decline in the CFUs of the inoculant in the rhizosphere was similar to that taking place in neighbouring surface soil. In the field, Thompson et al. [15] observed a better survival of *Flavobacterium* and *Arthrobacter* in the rhizosphere of winter and spring wheats than in unplanted soil, but van Elsas et al. [28] did not find any significant difference in the survival of *P. fluorescens* in bulk soil and in the rhizosphere of spring wheat over 120 days in field microplots. Here, the inoculant had no effect on the size of the total culturable population of fluorescent pseudomonads in the rhizosphere of winter wheat (Table 1). This was expected, because such quantitative effects are usually transient [12,29] and the first sampling was performed as long as 58 days after inoculation. The percentage of the total culturable fluorescent pseudomonads corresponding to CHA0-Rif decreased in time from December to July, both in soil [14] and in the rhizosphere (Table 1).

By the time spring wheat was sown (March 1994), the number of culturable cells of CHA0-Rif in surface soil had decreased from 8.7 (at inoculation, in October 1993) to 4.5 log CFU $(g\ soil)^{-1}$. However, the inoculant was found at similar CFUs in the rhizosphere of both wheat varieties, at flowering and at ripening (Tables 1 and 2). Colony counts for CHA0-Rif were low at ripening (Tables 1 and 2) and at two days after harvest (data not shown). In contrast, the survival of *P. fluorescens* SBW25EeZY-6KX on spring wheat roots for almost 300 days after harvest was high (>4 log CFU per g) but was not enhanced at later stages of plant development or during root decomposition after harvest [30].

As in the case of winter and spring wheats, the inoculant (added in April 1994) was found at low CFUs in the rhizosphere of maize (as well as in surface soil) in July 1994 (Table 3). This result is in accordance with the decrease in culturable cells of root-associated CHA0-Rif observed in natural soil microcosms planted with maize for 40 days after inoculation (Fig. 1). However, in the lysimeter experiment the number of culturable cells of CHA0-Rif in the rhizosphere of maize and the percentage of rhizosphere samples colonised by the pseudomonad increased at the later stages of plant development (Table 3). The colonisation of the rhizosphere of maize

by CHA0-Rif was in phase with that by the resident fluorescent pseudomonads and the inoculant represented 0.2-0.5% of the total culturable fluorescent pseudomonads (for roots colonised by CHA0-Rif) at all four samplings. This behaviour of pseudomonads has been overlooked in previous studies [10,31,32], essentially because determinations have not been performed over the whole crop cycle. Whether the absence of late colonisation of wheat roots by CHA0-Rif may be due in part to seasonal factors, since ripening does not happen at the same time for wheat (July) and maize (October), remains to be investigated. However, the results of the current work suggest that *Pseudomonas* inoculants, which have received attention as early, aggressive root colonisers have also potential for late colonisation of the rhizosphere of some crops, even if they are present at low CFUs in soil.

Certain pseudomonads can colonise the interior of the root [33,34], and the presence of CHA0 inside the roots (cortex and stele) of tobacco grown in sterile soil microcosms was shown by studying root sections in immunofluorescence microscopy [25]. In the current work, the introduced pseudomonad was found inside the roots of all three crops studied, by colony counts of surface-sterilised roots (Tables 1–3). Interestingly, the CFUs of CHA0-Rif in the interior of roots were usually as high (or even higher) as those in the rhizosphere and displayed essentially similar dynamics in time. Particularly striking is the observation that CHA0-Rif, which could not be detected inside the roots of maize in July 1994 (i.e. 91 days after inoculation of soil), was present at levels as high as 5 log CFU (g fresh root)$^{-1}$ at harvest in colonised roots. In comparison, CFUs of the inoculant inside the roots of winter and spring wheats (as well as in their rhizosphere) two days after harvest were similar to or lower than those at the previous sampling, carried out at ripening (data not shown). Furthermore, the culturable population of the inoculant inside the roots of maize increased by about two log units over a four-day period during ripening (end of October 1994; Table 3). Obviously, the maize rhizosphere effect on CHA0-Rif at later stages of plant development was less pronounced than the stimulation taking place inside the maize roots. It is likely that the late colonisation of maize roots by CHA0-Rif happened at the expense of nutrients released from decaying root tissues. However, the possible role of plant defence mechanisms or plant signal molecules cannot be ruled out. Root exudates of certain plants (but not others) can switch on specific genes in *P. fluorescens* [35]. Likewise, plant signals triggering a bacterial response have been documented for *Pseudomonas syringae* [36].

Interestingly, the cells of CHA0-Rif extracted from the interior of the roots of maize (at ripening) consisted of very long rods (7 μm) with flagella. Cells of CHA0-Rif that long have never been found under in vitro conditions (e.g. in overnight nutrient-yeast extract liquid cultures; Table 4) or within the root of tobacco [25], suggesting that the interior of the root of maize constitute a habitat particularly favourable for CHA0-Rif. However, the culturable cells of CHA0-Rif represented only 26% of the total number of cells of CHA0-Rif inside the roots of maize. The other cells of the strain were not distinguishable morphologically from the culturable cells (immunofluorescence microscopy) but proved to be viable (Kogure's DVC). Pseudomonads introduced into soil may lose culturability despite remaining viable [14,37]. In the current work, the presence of large numbers of viable but nonculturable cells of CHA0-Rif inside the roots of maize is somewhat surprising since the culturable population of the pseudomonad was undergoing a significant increase at the time of sampling (i.e. during ripening). Whether this increase resulted from actual growth of culturable cells present at very low levels inside the roots (or of culturable rhizoplane CHA0-Rif that had penetrated into the roots) and/or from the recovery of colony-forming ability by viable cells (as observed with *Vibrio* in marine microcosms; [38]) present inside the roots since the early stage of root colonisation in the spring is not known and deserves further study.

The colonisation of maize roots by CHA0-Rif and the resident fluorescent pseudomonads at later stages of plant development was reproduced in a soil microcosm experiment in which the shoots of half of the plants had been cut off. The roots started to decay under those experimental conditions and colony counts demonstrated that colonisation by CHA0-Rif and resident fluorescent pseudomonads was stimulated (Fig. 1), confirming that decaying maize roots may represent a favourable niche for

certain fluorescent pseudomonads, including CHA0-Rif.

In conclusion, the current investigation indicates that decaying root tissues may enable the persistence of introduced *Pseudomonas* inoculants at high CFUs after the harvest of certain crops, thereby possibly promoting their overwintering and subsequent colonisation of the following crop in the spring. From a biosafety point of view, these findings need to be taken into account when considering the monitoring of genetically-modified *Pseudomonas* inoculants in the field.

5. Unlinked References

[24]

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