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Survival and cell culturability of biocontrol *Pseudomonas fluorescens* CHA0 in the rhizosphere of cucumber grown in two soils of contrasting fertility status

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Abstract The effect of cucumber roots on survival patterns of the biocontrol soil inoculant *Pseudomonas fluorescens* CHA0-Rif was assessed for 22 days in two non-sterile soils, using a combination of total immunofluorescence cell counts, Kogure's direct viable counts and colony counts on plates containing rifampicin. In Eschikon soil (high fertility status for cucumber), CHA0-Rif persisted as culturable cells in bulk soil and in the rhizosphere, but colony counts were lower than viable counts and total cell counts inside root tissues. The occurrence of viable but non-culturable (VBNC) cells inside root tissues (5 log cells g^{-1} root) was unlikely to have resulted from the hydrogen peroxide treatment used to disinfect the root surface, as hydrogen peroxide caused the death of CHA0-Rif cells *in vitro*. In Siglistorf soil (low fertility status for cucumber), the inoculant was found mostly as non-culturable cells. Colony counts and viable counts of CHA0-Rif were similar, both in bulk soil and inside root tissues, whereas in the rhizosphere viable counts exceeded colony counts at the last two samplings (giving about 7 log VBNC cells g^{-1}). In conclusion, soil type had a significant influence on the occurrence of VBNC cells of CHA0-Rif, although these cells were found in root-associated habitats (i.e. rhizosphere and root tissues) and not in bulk soil.

Key words *Pseudomonas fluorescens* · Cucumber · Rhizosphere · Biocontrol · Culturability

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Introduction

The population dynamics of bacterial inoculants in soil systems is often studied by means of colony counts on selective media (Natsch et al. 1994; McInroy et al. 1996; Cronin et al. 1997). However, recent investigations showed that colony counts were insufficient to monitor bacterial inoculants in soil, as certain strains could persist as mixed populations of culturable and non-culturable cells (Binnerup et al. 1993; Heijnen et al. 1995; Troxler et al. 1997b). For instance, the biocontrol inoculant *Pseudomonas fluorescens* CHA0-Rif was found mostly as non-culturable cells in the surface soil horizon of large outdoor lysimeters, as indicated by the comparison of colony counts (on selective medium) and total immunofluorescence (IF) counts of the strain (Troxler et al. 1997b). Furthermore, a significant number of the non-culturable cells of the inoculant responded positively to Kogure's viability test (Kogure et al. 1979), indicating that they were not dead (Troxler et al. 1997b). Kogure's direct viable count (DVC) identifies substrate-responsive cells after incubation in the presence of nutrients and nalidixic acid, which prevents cell division and results in the enlargement of viable cells.

Several studies have focused on the culturability of *P. fluorescens* in bulk soil (Binnerup et al. 1993; Troxler et al. 1997b; Hase et al. 1999). However, whether or not root-associated pseudomonads can occur as non-culturable cells has received little attention so far. All cells of strain CHA0-Rif present inside maize roots at crop ripening responded positively to Kogure's viability test, but only a quarter of them could be recovered on solid medium (Troxler et al. 1997c). Only circumstantial evidence is available regarding the presence of non-culturable pseudomonads in the rhizosphere (Troxler et al. 1997b). This is an important issue, because pseudomonads are typical rhizobacteria (Miller et al. 1989; de Leij et al. 1995) and several *Pseudomonas* strains represent promising biocontrol agents against soil-borne patho-

gens affecting plant roots (Cook et al. 1995; Dunne et al. 1997; Sharifi-Tehrani et al. 1998).

The objective of this work was to assess the survival of the biocontrol agent *P. fluorescens* CHA0-Rif and its ability to persist as non-culturable cells in the rhizosphere. Two soils were used, since colony counts (van Elsas et al. 1986) and the occurrence of non-culturable cells (van Overbeek et al. 1995) of *P. fluorescens* introduced into bulk soil can vary greatly from one type of soil to the next. Furthermore, the effect exerted by plant roots on culturable soil fluorescent pseudomonads also depends on soil type (Latour et al. 1996). Soil or rooting medium conditions influence the growth of roots and the release of root exudates, including that of exudated compounds used as nutrients or recognised as signals by soil bacteria (Prikryl and Vancura 1980; Richardson et al. 1988; Marschner 1991; Buerkert and Marschner 1992). Therefore, two soils displaying different physical and chemical characteristics and representing contrasting fertility status for cucumber were chosen. One of the soils (Eschikon soil) was a loam, in which cucumber grows well, and the other one (Siglistorf soil; acidic silt with a high organic matter content) corresponds to a type of soil in which cucumber grows slowly (Papadopoulos 1994). *P. fluorescens* CHA0-Rif was inoculated into the soils, which were then sown with cucumber. The inoculant was monitored in bulk soil, in the rhizosphere and inside root tissues using a combination of colony counts, Kogure's DVCs (Kogure et al. 1979) and total IF counts of the strain.

Materials and methods

Soils used in the experiment

Both soils were obtained from the surface horizon of Cambisols. The first soil (described by Natsch et al. 1994) was collected at a fallow located in Eschikon (near Zurich) and corresponds to a loam (15% clay, 42% silt, 43% sand; pH 7.0, 3.5% organic matter). The second soil was obtained in a forest located near Siglistorf and corresponds to a silt (16% clay, 53% silt, 31% sand; pH 4.3, 10% organic matter). Plant roots were removed. The soils were passed through a 5-mm-mesh screen and air-dried at 15 °C until the water potential (determined by the filter paper method; McInnes et al. 1994) reached approximately −0.03 MPa (i.e. water contents of 22.5% w/w and 31.6% w/w for Eschikon and Siglistorf soils, respectively).

Bacterial strains, inoculation of soil and sowing

The biocontrol agent *P. fluorescens* CHA0 was isolated from the roots of tobacco grown in Morens soil (near Fribourg, Switzerland; Stutz et al. 1986). Strain CHA0-Rif is a spontaneous rifampicin-resistant mutant of *P. fluorescens* CHA0 (Natsch et al. 1994).

Strain CHA0-Rif was routinely grown at 27 °C with shaking [150 revolutions (rev) min^{-1}] in King's B broth (King et al. 1954) containing 100 μg rifampicin ml^{-1} (Rif100). The cells were used to inoculate King's B agar (KBA) containing Rif100 and the plates were incubated overnight at 27 °C. The cells were harvested from the plates, washed 3 times with sterile distilled water and the cell suspension was adjusted to 5×10^9 cells ml^{-1} based on optical density measurements (600 nm). Bacteria were applied to the soil by spraying 20 ml suspension dm^{-3} of soil (i.e. about 2×10^8 cells g^{-1}). In the uninoculated control, soil was sprayed with 20 ml sterile distilled water dm^{-3} soil. The soil was thoroughly mixed and 500 cm^3 soil (i.e. dry weight of 410 g Eschikon and 220 g Siglistorf) was added per pot.

Cucumber seeds (*Cucumis sativus* L. "Sensation"; Geissler, Zürich) were surface-disinfected in 5% (w/v) sodium hypochloride for 30 min and rinsed with sterile distilled water. One seed was sown per pot, immediately after soil inoculation. The pots were placed in a growth chamber (70% relative humidity; 16 h at 160 μE m^{-2} s^{-1} at 22 °C and 8 h dark at 18 °C), in a randomised design. Every second day, the water content of the soil was checked by weighing the pots and, if necessary, adjusted to reach about −0.03 MPa by adding sterile distilled water to the soil surface. Fresh root biomass was 1.22 g (Eschikon) and 0.071 g $plant^{-1}$ (Siglistorf) at 22 days, confirming that the latter soil has a low fertility status for cucumber. Roots were healthy (no disease symptoms) in both soils.

Sampling of microcosms and monitoring of the inoculant

Destructive sampling was done at 2, 7, 14 and 22 days. The first 5 mm soil in the pots was removed with a sterile spatula and discarded. Bulk soil was collected (5 g pot^{-1}). Plants were harvested and loosely adhering soil was shaken off. Rhizosphere samples consisted of germinated seeds (at 2 days) or root systems (at subsequent samplings) with closely adhering soil. All samples were agitated in 1 ml (seeds) or 50 ml (roots and 5-g bulk soil samples) sterile distilled water for 15 min at 300 rev min^{-1}. Dilution series of the extracts were prepared in sterile distilled water. At 22 days, the procedure was completed by a subsequent treatment of the roots with 10% hydrogen peroxide (15 s), which were then rinsed 3 times in 50 ml sterile distilled water. This treatment effectively disinfected the root surface, as indicated by the absence of colonies when the last water used to rinse the roots was afterwards spread plated on S1 agar (Gould et al. 1985). Surface-disinfected roots were ground aseptically in 10 ml sterile distilled water, using a mortar and pestle, and cells present in root tissues were extracted by shaking the resulting samples (in 28-ml Universal bottles; 30 min at 350 rev min^{-1}; Troxler et al. 1997c).

Culturable cells of CHA0-Rif were enumerated by counting colonies on KBA containing Rif100, after having incubated the plates for 48 h at 27 °C (two plates per dilution). No colony was found when samples from uninoculated microcosms were plated [detection limits of 10^3 colony-forming units (CFU) g^{-1} soil, 4.6×10^3 CFU g^{-1} seed and 2.5×10^3 CFU g^{-1} root]. Viable cells of CHA0-Rif were quantified using the DVC technique of Kogure et al. (1979), in which enlarged cells were counted by immunofluorescence microscopy, as described by Troxler et al. (1997b) and Hase et al. (1999). The primary antiserum used is specific for CHA0 (Troxler et al. 1997a) and no cross-reaction was found here when examining samples from uninoculated microcosms. In brief, samples were incubated for 6 h in the presence of yeast extract (250 μg ml^{-1} sample) and nalidixic acid (20 μg ml^{-1} sample), and were fixed with formaldehyde (20 mg ml^{-1} sample). Nalidixic acid inhibits cell division in CHA0-Rif (Troxler et al. 1997b; Hase et al. 1999). The samples were passed through 0.2-μm (pore size) polycarbonate filters stained with Irgalan Black (Hobbie et al. 1977). The filters were treated successively with the primary antiserum (60 min) and the secondary antibody (fluorescein isothiocyanate-conjugated goat anti-rabbit immunoglobulins; 45 min) prior to treatment with 1,4-diazobicyclo-(2,2,2)-octan-glycerol mounting medium to prevent fading (Johnson et al. 1982). Cells of CHA0-Rif were counted using a Zeiss Axioskop epifluorescence microscope (filters 450–490 nm; at least 20 fields and/or 150 bacterial cells $sample^{-1}$). Elongated cells (length >3 μm) were considered as viable cells. The same filters were used to count also the total number of cells of CHA0-Rif.

Effect of hydrogen peroxide on the inoculant

Since hydrogen peroxide was used to disinfect the surface of root samples, the effect of the compound on *P. fluorescens* CHA0-Rif was assessed under in vitro conditions. Cells of the inoculant were prepared, as described above (about 8×10^9 cells ml^{-1}), and exposed to hydrogen peroxide (0.1–10% hydrogen peroxide) for 15 s. Total IF counts, DVCs and colony counts of the pseudomonad were determined as described above.

Statistics

Each treatment was replicated 3 times in soil microcosms (i.e. three microcosms studied by destructive sampling at each sampling time) and when the strain was exposed to hydrogen peroxide *in vitro*. Cell counts were log-transformed. Data from soil microcosms were analysed at each sampling time, using two-factor (cell count method × soil type) ANOVA, followed (when appropriate) with Fisher's least significant difference (LSD) test. Similarly, data from the in vitro experiment were analysed using a two-factor (cell count method × hydrogen peroxide concentration) ANOVA and Fisher's LSD test. All statistical analyses were conducted at $P < 0.05$ using version 5.0 of SYSTAT for Windows (SYSTAT, Evanston, Ill.).

Results and discussion

Survival and cell culturability of the inoculant in bulk soil

When Eschikon soil was used, cell numbers of *P. fluorescens* CHA0-Rif in bulk soil decreased from 8 log cells g^{-1} to approximately 6.5 log cells g^{-1} soil in the 22-day experiment, regardless of the method used to count the cells (Fig. 1A). This result is in accordance with previous findings (Hase et al. 1999). In contrast, total IF counts of CHA0-Rif in bulk Siglistorf soil remained at levels of 7.6 log cells g^{-1} soil (or higher), and they exceeded the other cell counts throughout the experiment (by more than 1 log unit at 22 days; Fig. 1A). DVCs and colony counts of the pseudomonad were essentially similar at each sampling in this soil.

Despite not responding to Kogure's viability test, the non-culturable cells found in bulk Siglistorf soil were probably not dead, as most heat-killed bacterial cells tend to disappear rapidly once added to non-sterile soil (Cleyet-Marel and Crozat 1982; Turpin et al. 1993). Non-culturable cells of *P. fluorescens* R2f Rp^r occurred in Ede loamy sand, but not in Flevo silt loam (van Overbeek et al. 1995). As in the latter study, several characteristics differed between the two soils in the current investigation (i.e. texture, pH, etc.), and further work will be necessary to identify soil properties responsible for the different behaviour of CHA0-Rif. For instance, bulk soil density at 22 days was higher for Eschikon soil (i.e. 0.91) than for Siglistorf soil (i.e. 0.49), which perhaps influenced strain survival. Viable but non-culturable (VBNC) cells of the inoculant (i.e. cells that did not form a colony on the plate but responded positively to a viability test) were not found in bulk soil here, but have been reported in non-rhizosphere soil:

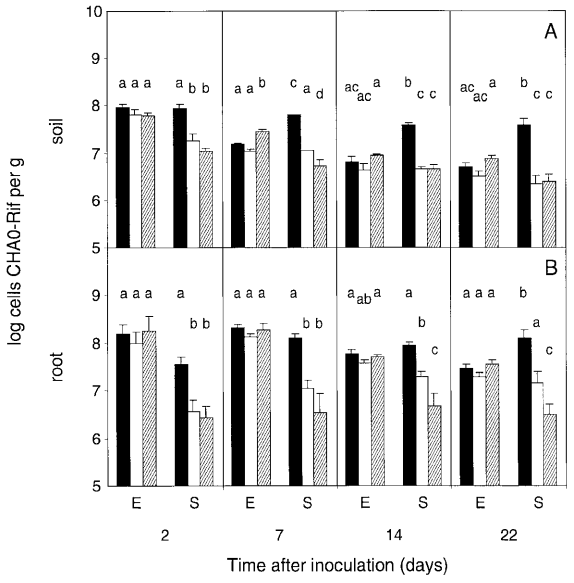

Fig. 1 Influence of soil type on the survival of *Pseudomonas fluorescens* CHA0-Rif in bulk soil (**A**) and in the rhizosphere of cucumber (**B**). In both soils, the inoculant was added at 8.1 log cells g^{-1}. The strain was monitored by total immunofluorescence (IF) counts (*black bars*), direct viable counts (DVCs; *white bars*) and colony-forming units (CFUs; *hatched bars*). *Error bars* represent SEs. At each sampling time, data were analysed separately in A and in B. Different letters (*a*, *b* and *c*) indicate statistically significant differences at $P < 0.05$

(1) for other bacteria including a pseudomonad (Binnerup et al. 1993) or (2) when CHA0-Rif was exposed to abiotic stress in the surface horizon of lysimeters (Troxler et al. 1997b).

Survival and cell culturability of the inoculant in the rhizosphere

In the rhizosphere of cucumber grown in Eschikon soil, CHA0-Rif was recovered at levels between 7.3 log cells g^{-1} root and 8.3 log cells g^{-1} root during the 22-day experiment, and no difference was found at any of the samplings between total IF counts, DVCs and CFUs of the strain (Fig. 1B). Likewise, total IF counts, DVCs and CFUs of *Flavobacterium* sp. P25 were similar in the rhizosphere of wheat in Ede loamy sand (Heijnen et al. 1995). In Siglistorf soil however, total IF counts of CHA0-Rif were higher than DVCs and CFUs throughout the experiment (as in bulk soil), and DVCs exceeded CFUs at the last two samplings. Total IF counts were lower at 2 days than at subsequent samplings. This could be due to the fact that germination was slower in that soil, with the likely consequences that exudation was lower and that the amount of soil adhering to the plant at 2 days was less than that at the following samplings.

The occurrence of VBNC cells of CHA0-Rif in the rhizosphere but not in bulk soil in Siglistorf microcosms

suggests that the presence of roots favoured the maintenance of substrate-responsiveness in the subpopulation of cells undergoing a loss in culturability. An alternative hypothesis is that the root itself could be involved in the loss of colony-forming ability, resulting in the formation of VBNC cells. Indeed, unfavourable growth conditions for cucumber, linked to the physicochemical properties of this soil (Papadopoulos 1994), resulted probably in altered patterns of root exudation compared with those in Eschikon soil. How this could have resulted in the occurrence of VBNC cells of CHA0-Rif in the rhizosphere in Siglistorf microcosms remains to be ascertained.

Survival and cell culturability of the inoculant inside the root

Strain CHA0-Rif can behave as a root endophyte (Troxler et al. 1997a), and VBNC cells of the strain were found inside roots of field-grown maize at harvest time (Troxler et al. 1997c). In the current work, total IF counts and DVCs of the strain exceeded CFUs in root tissues when Eschikon soil was used (Fig. 2). Thus, VBNC cells of CHA0-Rif occurred only inside the root in Eschikon microcosms. In contrast, total IF counts were higher than both DVCs and CFUs in root tissues of cucumber grown in Siglistorf soil (Fig. 2). In the latter microcosms, VBNC cells were not found inside the root despite occurring in the rhizosphere.

Hydrogen peroxide, which was used to surface-disinfect root samples, had no effect on cell numbers of CHA0-Rif when the strain was exposed to 0.1% or 1% hydrogen peroxide in vitro, and cells remained culturable (Fig. 3). After a 15 s incubation of CHA0-Rif in 10% hydrogen peroxide, however, total IF counts were still at the inoculation level, whereas DVCs and CFUs were below the detection limit (2.0 log cells ml^{-1} for both). The actual concentration of hydrogen peroxide inside the root during surface disinfection of samples was unlikely to reach this level, but nonetheless this observation raises the possibility that, in this and other studies (Troxler et al. 1997c), certain bacterial cells present in the root cortex can be exposed to hydrogen peroxide levels sufficiently high to result in a loss of colony-forming ability. In the current work, however, the hydrogen peroxide treatment of samples was unlikely to explain the occurrence of VBNC cells of CHA0-Rif inside roots grown in Eschikon soil, as exposure of the strain to hydrogen peroxide in vitro did not result in VBNC cells.

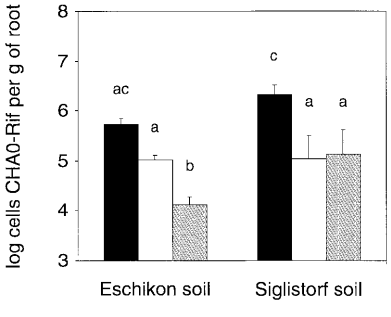

Fig. 2 Influence of soil type on cell numbers of *P. fluorescens* CHA0-Rif inside root tissues at 22 days after sowing. The strain was monitored by total IF counts (*black bars*), DVCs (*white bars*) and CFUs (*hatched bars*). *Error bars* represent SEs. Different letters (*a*, *b* and *c*) indicate statistically significant differences at $P<0.05$

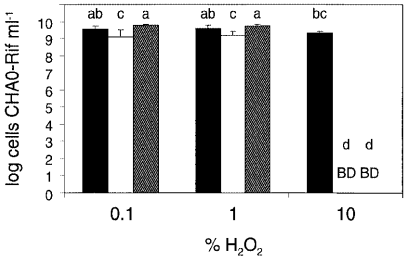

Fig. 3 Effect of hydrogen peroxide (H_2O_2) on cell numbers of *P. fluorescens* CHA0-Rif in vitro. The strain was added at 9.9 log cells ml^{-1} and monitored by total IF counts (*black bars*), DVCs (*white bars*) and CFUs (*hatched bars*) 15 s after exposure to H_2O_2 Colony counts on King's B agar (KBA; data not shown) and KBA plus 100 μg rifampicin ml^{-1} were identical. *Error bars* represent SEs. Statistics were done to compare the nine treatments (an arbitrary log value of zero was used when counts were below the detection limit), and different letters (*a*, *b* and *c*) indicate statistically significant differences at $P<0.05$. *BD* Below detection limit (2.0 log cells ml^{-1})

Several rifampicin-resistant pseudomonads recovered from root tissues of cucumber failed to grow on medium containing rifampicin, but could do so once previously cultured on rifampicin-free medium (i.e. rifampicin-resistance masking; McInroy et al. 1996). Here, colony counts of CHA0-Rif on KBA and KBA plus Rif100 were identical when studying root tissue samples obtained from cucumber grown in sterile Eschikon soil (data not shown), indicating that the presence of rifampicin in plates could not account for the non-culturability of the VBNC cells mentioned above (Fig. 2). The fact that DVCs and CFUs of CHA0-Rif inside root tissues were statistically identical in the case of Siglistorf soil is further evidence that this pseudomonad does not display masking of rifampicin resistance.

In conclusion, when Eschikon soil was used, *P. fluorescens* CHA0-Rif persisted as culturable cells in bulk soil and in the rhizosphere of cucumber, but VBNC cells of the strain were found inside root tissues. In contrast, non-culturable cells of CHA0-Rif were identified in bulk soil, in the rhizosphere and inside root tissues of cucumber when Siglistorf soil was used, and VBNC cells of the inoculant were found in the rhizosphere at the last two samplings. Therefore, the occurrence of non-culturable cells of CHA0-Rif was strongly influenced by the soil type, even in root-associated habitats (i.e. rhizosphere and root tissues), and VBNC cells were found only in the latter habitats.

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