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ORIGINAL PAPER

Survival of native *Pseudomonas* in soil and wheat rhizosphere and antagonist activity against plant pathogenic fungi

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Abstract Survival of *Pseudomonas* sp. SF4c and *Pseudomonas* sp. SF10b (two plant-growth-promoting bacteria isolated from wheat rhizosphere) was investigated in microcosms. Spontaneous rifampicin-resistant mutants derived from these strains (showing both growth rate and viability comparable to the wild-strains) were used to monitor the strains in bulk soil and wheat rhizosphere. Studies were carried out for 60 days in pots containing non-sterile fertilized or non-fertilized soil. The number of viable cells of both mutant strains declined during the first days but then became established in the wheat rhizosphere at an appropriate cell density in both kinds of soil. Survival of the strains was better in the rhizosphere than in the bulk soil. Finally, the antagonism of *Pseudomonas* spp. against phytopatogenic fungi was evaluated in vitro. Both strains inhibited the mycelial growth (or the resistance structures) of some of the phytopathogenic fungi tested, though variation in this antagonism was observed in different media. This inhibition could be due to the production of extracellular enzymes, hydrogen cyanide or siderophores, signifying that these microorganisms might be applied in agriculture to minimize the utilization of chemical pesticides and fertilizers.

Keywords Biocontrol · PGPRs · *Pseudomonas* · Survival

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Introduction

The multiplying population is exerting immense pressure on agricultural lands for more crop yields, which results in more and more extensive use of chemical fertilizers. These agents are costly and create environmental problems; consequently, there has recently arisen a renewed interest in environmentally friendly agricultural practices (Karlidag et al. 2007). Accordingly, non-pathogenic soil bacteria having the ability to antagonize fungal phytopathogens and thus promote plant growth represent an attractive alternative to substitute agrochemicals. Plant Growth-Promoting Rhizobacteria (PGPRs) may stimulate host-plant growth: (1) directly, by increasing nutrient and water uptake and thus enhancing plant biomass, through the production of phytohormones (e.g., Indole-3-acetic acid), siderophores, organic acids involved in phosphorus solubilization, or the fixation of atmospheric N_2 (O'Sullivan and O'Gara 1992; Pattern and Glick 1996; Lugtenberg and Kamilova 2009). Many

PGPRs also contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, a hydrolase of ACC (the precursor of ethylene in plant) and thus in this way reduce the level of ethylene during plant development (Glick 1995). (2) indirectly, by producing secondary metabolite (such as antibiotics, hydrogen cyanide) that inhibit soil pathogens or by synthesizing cell-wall-degrading enzymes of phytopahogens or by competing for colonization sites, nutrients, etc. (Whipps 2001). For example, in the case of the secondary metabolites, 2,4-Diacetylphloroglucinol (DAPG), phenazine-1-carboxylic acid (PCA), Pyrrolnitrin (PRN) and hydrogen cyanide (HCN) play a key role in the suppression of plant pathogens by several strains of *Pseudomonas*. Genes enconding these metabolites have been characterized and specific primers designed for their detection (Raaijmakers et al. 1997; De Souza and Raaijmakers 2003; Svercel et al. 2007).

Because of their catabolic versatility, excellent root-colonizing abilities, and capacity to produce a wide range of antifungal metabolites, the soil-borne fluorescent *Pseudomonas* had received particular attention among PGPRs (Walsh et al. 2001).

The chances for selecting effective strains of bioinoculants are likely to improve when the microorganisms are isolated from the same environment where they would ultimately be required to work (Whipps 1997; Pandey et al. 1998). Taking this into account, we have previously obtained a collection of strains from the rhizosphere of wheat grown in fields of the Córdoba′s province (Argentina). These native strains promote wheat growth under greenhouse conditions probably because they present some PGPR traits (production of Indole-*3*-acetic acid-like compounds or siderophore or the solubilization of phosphate). One such strain was identified as belonging to the *Pseudomonas* genus and designated *Pseudomonas* sp. SF4c (Fischer et al. 2007).

On the other hand, little work has been done concerning the survival and persistence of native strains in Argentinean soil. The successful use of native strains of *Pseudomonas* as bioinoculants for applications on crops requires these strains to compete satisfactorily with the indigenous microflora of soil, to survive, and to persist in adequate viable-cell numbers in the rhizosphere. The aim of this study was therefore to evaluate survival and persistence of native *Pseudomonas* strains in bulk soil and wheat rhizosphere; their ability to promote plant growth in other hosts such as tomato and their capability of inhibiting the growth of phytopathogenic fungi.

Materials and methods

Strains and growth conditions

Pseudomonas sp. SF4c, *Pseudomonas* sp. SF10b and *Pseudomonas* sp. SF39a are native strains isolated from wheat root grown in the soil of Córdoba's province (Fischer et al. 2007). *Pseudomonas* sp. SF4cR and *Pseudomonas* sp. SF10bR are spontaneous rifampicin-resistant mutants derived from SF4c and SF10b, respectively. Native and reference strains of *Pseudomonas* were maintained in nutrient agar and kept at −80°C in 40% glycerol.

PCR amplification with *Pseudomonas*-specific primers

PCR reaction was performed with the *Pseudomonas*-specific primers PsF and PsR described by Widmer et al. (1998). The amplification was carried out in a 15 µl reaction mixture contained 1.5 µl of 10× PCR buffer [200 mM Tris–HCl (pH 8.4), 500 mM KCl], 1.5 mM $MgCl_2$, 0.13 mM of each of the four dNTPs, 0.8 µM of each primers, 0.75 U *Taq* DNA polymerase (Invitrogen Life Technologies) and 3 µl of DNA. Reference strains of *Pseudomonas* were used as a positive control. The amplification was performed with a Techne TC 312 Thermal Cycler at 95°C for 5 min, followed by 30 cycles of 94°C for 45 s, 66°C for 1 min, and 72°C for 1 min, and a final extension step at 72°C for 10 min. PCR products were run on a 0.8% agarose gels.

Sequences of 16S rDNA and phylogenetic tree

SF10b and SF39a isolates were sent to Macrogen Inc. for sequencing of 16S rDNA. Homology studies were carried out with the NCBI GenBank BLAST program (Altschul et al. 1997). The sequences of the isolates SF10b and SF39a were aligned, by means of clustal W, with the sequences retrieved from the GenBank database. The construction of neighbor-joining tree (Saitou and Nei 1987) and bootstrap analysis of 1,000 resamplings (Felsenstein 1985) were performed.

Phylogenetic analyses were conducted in MEGA4 (Tamura et al. 2007).

The sequences obtained were deposited in GenBank nucleotide-sequence database under the accession number AY870438 and EU882159.

Amplified ribosomal DNA restriction analysis (ARDRA)

Full-length 16S rRNA gene fragments from strains SF4c, SF10b and SF39a and from reference strains of *Pseudomonas* were obtained by PCR amplification with primers rD1 and fD1 (Weisburg et al. 1991). The reaction was performed in a 50 µl reaction mixture, with the following programs: initial denaturation at 95°C for 3 min; 35 cycles at 94°C for 40 s, 55°C for 40 s and 72°C for 2 min and final extension at 72°C for 10 min. The PCR mixture contained 5 µl of 10× PCR buffer, 1.5 mM $MgCl_2$, 0.2 mM of each of the four dNTPs, 0.5 µM of each primers, 2.5 U *Taq* DNA polymerase (Invitrogen Life Technologies) and 13.25 µl of DNA.

Ten microlitre of PCR products were digested overnight at 37°C with 5 U restriction endonucleases *Hae*III, *Rsa*I, *Taq*I, *Cof*I and *Hinf*I. Digests were subjected to electrophoresis in 2% agarose gels. A binary-data matrix was constructed on the basis of the presence or absence of each band (scored 1 or 0, respectively) generated by the digestion of amplified 16S rDNA. A dendrogram based on the unweighted pair groups mean average method (UPGMA) clustering analysis of the Jaccard similarity coefficient was constructed.

Soil characteristics

The soils used in this study were collected from fields from Alejandro Roca (Córdoba's province) in two conditions (with and without prior fertilization with 130 kg ha^{-1} urea). Surface soil (<30 cm) was collected and sieved through a 2-mm mesh and stored in plastic bags at 4°C. The characteristics of the non-fertilized soil were: organic content (1.57%), pH = 6.85, extractable phosphorus (24.5 mg kg^{-1} soil), nitrogen of nitrate (6.2 mg kg^{-1}). Fertilized soil: organic content (2.24%), pH = 5.72, extractable phosphorus (14.40 mg kg^{-1}), nitrogen of nitrate (15.2 mg kg^{-1}).

Competitiveness assays

Spontaneous rifampicin-resistant mutants derived from SF4c and SF10b were isolated on Luria–Bertani (LB) medium containing rifampicin (100 µg ml^{-1}). Studies of growth and competition of the wild-type strain and their spontaneous rifampicin-resistant mutants (SF4cR and SF10bR) were carried out in liquid medium to assess their competitive abilities. Parent and rifampicin-resistant strains were inoculated in glucose minimal medium (GMM) and LB liquid medium alone (pure culture) or together (mixed culture), with the same initial viable-cells number. The colony-forming units (cfu) were determined on an appropriate solid medium; rifampicin (100 µg ml^{-1}) was added when required. For mixed culture, the cell number corresponding to the wild-type strain was calculated by subtracting the number of antibiotic-resistant cells (cfu from medium with rifampicin) from the total number (cfu from medium without rifampicin) (Duquenne et al. 1999).

Survival of native *Pseudomonas* in soil and wheat rhizosphere

Persistence of *Pseudomonas* sp. SF4c and *Pseudomonas* sp. SF10b in soil and in the wheat rhizosphere and their competitiveness against indigenous microflora was determined in non-sterile soil.

Wheat seeds (*Triticum aestivum* cv Guatimozin) were surface sterilized and pregerminated (Fischer et al. 2007). Germinated seeds were sown into plastic pots containing non-sterilized fertilized or non-fertilized soil and then kept in a greenhouse with light–dark cycles (16 h light, 8 h dark). Plants were irrigated with sterilized water. Uninoculated plants were used as a control.

For production of bacterial inocula, the strains SF4cR and SF 10bR were grown in GMM broth at 30°C, 140 rev min^{-1} to until exponential phase (OD_{600} = 0.9 for SF4c or OD_{600} = 0.5 for SF10b). Cells were then harvested by centrifugation (9000 rev min^{-1} for 10 min), washed in physiological solution and resuspended in the same buffer at a density of 1×10^8 cfu ml^{-1}. One millilitre of inoculum was added to 7-day-old plants to a density of 1×10^8 cfu ml^{-1} per plant (equivalent to 1×10^6 cfu g^{-1} soil).

The plants were harvested at 15, 30 and 60 days after inoculation and the number of viable cells was determined in the rhizosphere soil, the root and in bulk soil (van Overbeek et al. 1997; Molina et al. 2000). Samples were serially diluted and plated onto nutrient agar supplemented with rifampicin (100 μg ml^{-1}) and cycloheximide (100 μg ml^{-1}). The plates were incubated at 30°C. Viability was expressed as cfu per g of dry soil (rhizosphere and bulk soil) or as cfu per g of fresh root (rhizoplane).

Growth promotion in tomatoes

Seeds from tomatoes (*Lycopersicon esculentum* cv pitenza) were surface sterilized and pregerminated in Petri dishes containing agar 0.7%. Seedlings were transferred to trays containing vermiculite:peat (1:1) and then kept in a greenhouse with light–dark cycles (14 h light at 24°C, 10 h dark at 20°C). After 4 days, 1 ml of inoculums was added to each plant at a density of 5×10^8 cfu ml^{-1}.

To produce the inocula, bacteria were grown on nutrient agar plates for 24 h at 30°C. The cells were then collected and resuspended in physiological solution at a final density of 5×10^8 cfu ml^{-1}.

After 2 weeks, plants were inoculated again with 5 ml per plant (5×10^8 cfu ml^{-1}). The plants were then irrigated with water and finally harvested 15 days after the second inoculation. The dry weight of the shoot and root and diameters of the neck were determined.

Statistical analyses

Data were analyzed by one way analysis of variance (ANOVA) or Student test when suitable. Differences were considered to be significant at the $P < 0.05$ level. The means were compared by Fisher's protected LSD (for ANOVA).

Antifungal activity of native *Pseudomonas*

The antagonistic activity of native *Pseudomonas* spp. against phytopatogen fungi (*Fusarium verticilloides* RC2000, *Fusarium solani*, *Fusarium graminearum* RC 664, *Fusarium proliferatum* RC 479, *Rhizoctonia solani, Sclerotinia minor, Sclerotinia sclerotiorum, Sclerotium rolfsii*) were tested on potato dextrose agar (PDA) and trypticase-soya-agar (TSA) media (McSpadden et al. 2005). Incubation was at room temperature and all fungi were also grown on PDA and TSA alone as a control. *Sclerotinia* and *Fusarium* were kindly supplied by the laboratories of Phytopathology and Mycology, Universidad Nacional de Río Cuarto, respectively.

Native strains were tested for their ability to produced lytic enzymes. Protease activity was determined in medium containing 3% skim milk; β-glucanase activity assayed in plates containing 0.1% lichenan (Sigma) (Walsh et al. 1995); cellulose activity in medium with 2% carboxymethylcellulose (Hankin and Anagnostakis 1977) and chitinase activity in medium containing 0.15% colloidal chitin (Shimahara and Takiguchi 1988). The plates were observed for degradation zones judged by clearing after 1–7 days of growth at 30°.

Detection of antibiotic and hydrogen cyanide encoding genes in *Pseudomonas*

A PCR assay was carried out to detect the *phl*D and *phz* genes [involved in the biosynthesis of DAPG and PCA, respectively] in native *Pseudomonas* through the use to the primers and protocols described by Raaijmakers et al. (1997). In addition, a PCR assay involving the specific primers PRND1 and PRND2 was performed as described by De Souza and Raaijmakers (2003) to detect *prn*D, gene encoding the production of PRN. *Pseudomonas* sp. Phz24 (producer of PRN and PCA) and *Pseudomonas* sp. P60 (producer of DAPG) were used as positive controls.

In addition, a PCR reaction was performed with specific primers for the detection of *hcnBC* genes (involved in the biosynthesis of HCN synthetase critical for HCN production) as described Svercel et al. (2007). *P. fluorescens* CHA0 was used as positive control. Ten microlitres of PCR products were run on a 0.8% agarose gels.

Results

PCR amplification with *Pseudomonas*-specific primers

Specific primers for the amplification of *Pseudomonas* 16S-rRNA gene have been designed and

represent a powerful tool for detection of bacteria belonging to this genus (Widmer et al. 1998). Accordingly, isolates previously obtained from the wheat rhizosphere and characterized as PGPRs were subjected to PCR amplification through the use of these *Pseudomonas*-specific primers (Widmer et al. 1998). A single fragment of approximately 1,000 bp was observed in two isolates (SF39a and SF10b) as well as in the reference strains *P. putida* KT2440, *P. fluorescens* WCS365 and the native *Pseudomonas* sp. SF4c, isolated in our laboratory and previously identified as *Pseudomonas* spp. (Data not shown).

Sequences of 16S rDNA and phylogenetic tree

The sequencing of 16S rDNA from the isolates SF10b and SF39a was performed to confirm the results obtained above. In order to find the most similar available sequences, a BLAST search was done in the NCBI databases. The sequence of 16S rDNA from both strains showed an identity of 99–100% with sequences belonging to the *Pseudomonas* genus. SF4c had also been previously identified as a member of pseudomonad, by sequencing the 16S rDNA (Fischer et al. 2007).

The nucleotide sequences corresponding to the 16S-rRNA gene from the native strains were aligned, by means of Clustal W, with 16S-rDNA sequences from *Pseudomonas* spp. and from soil bacteria of other genera retrieved from the GenBank database. Fig. 1 shows the phylogenetic tree based on 16S-rDNA sequences constructed by the neighbor-joining method. Cluster analysis indicated a close evolutionary relationship among the native strains (SF39a and SF10b) and those of the *Pseudomonas* genus.

Amplified ribosomal DNA restriction analysis (ARDRA)

A fragment of 1.5 kb corresponding to the 16S rDNA from the strains SF10b, SF4c and SF39a was amplified with the primers fD1 and rD1. The PCR product was digested with 5 endonucleases (*Rsa*I, *Hinf*I, *Hae*III, *Taq*I, *Cof*I). The restriction patterns of the native strains were compared with those obtained from the reference strains, *P. putida* KT2440, *P. fluorescens* WCS365, *P. syringae* pv. *syringae*. The digestion with the endonucleases *Cof*I and *Hae*III yielded identical profile among the native strains, *P. putida* KT2440 and *P. fluorescens* WCS365; while the restriction with *Rsa*I, *Hinf*I and *Taq*I resulted in a polymorphic banding pattern. Native *Pseudomonas* spp. were grouped in two clusters: the first contained the strains SF39a and SF4c plus *P. fluorescens* WCS365 at a similarity level of about 80%, the strain SF10b fell into the second along with *P. putida* KT2440 at a similarity level of more than 90% (Fig. 2). Therefore, we could conclude that SF39 and SF4c are closely related and consequently the following experiments were carried out only with strains SF4c and SF10b.

Competitiveness assays

To monitor the survival of *Pseudomonas* sp. SF4c and *Pseudomonas* sp. SF10b in soil, we first obtained spontaneous rifampicin-resistant mutants (SF4cR and SF10bR, derived from SF4c and SF10b, respectively). A series of experiments was carried out in order to determine whether spontaneous rifampicin-resistant mutants were able to grow the same as their parent strains. The number of viable wild-type and mutant cells were similar in either LB medium or GMM when the bacteria were grown in either pure or mixed culture (Table 1). Thus, spontaneous rifampicin-resistant mutants are as competitive in growth as their parent strains. For this reason, these strains could be used to monitor the survival and persistence of bacteria in wheat rhizosphere and in soil.

Survival and persistence of *Pseudomonas*

Survival and competitiveness of *Pseudomonas* sp SF4cR and *Pseudomonas* sp SF10bR were monitored for 60 days in wheat plants grown in pots containing non-sterile fertilized or non-fertilized soil.

In fertilized rhizosphere soil, the number of viable SF4cR cells was reduced from $\log_{10}$ 6 cfu g^{-1} of soil (initial density) to $\log_{10}$ 5.23 and 4.67 cfu g^{-1} of soil at 15 and 30 days, respectively. Subsequently, the cell density of this strain remained relatively constant over time (4.52 cfu g^{-1} soil at 60 days). In fertilized bulk soil, the number of these bacteria was lower than in the rhizosphere and declined below the detection limit (fewer than 3×10^3 cfu g^{-1}) at day 60 (Fig. 3a).

The viable-cell number of SF10bR strain likewise declined in the rhizosphere from $\log_{10}$ 6 cfu g^{-1} of

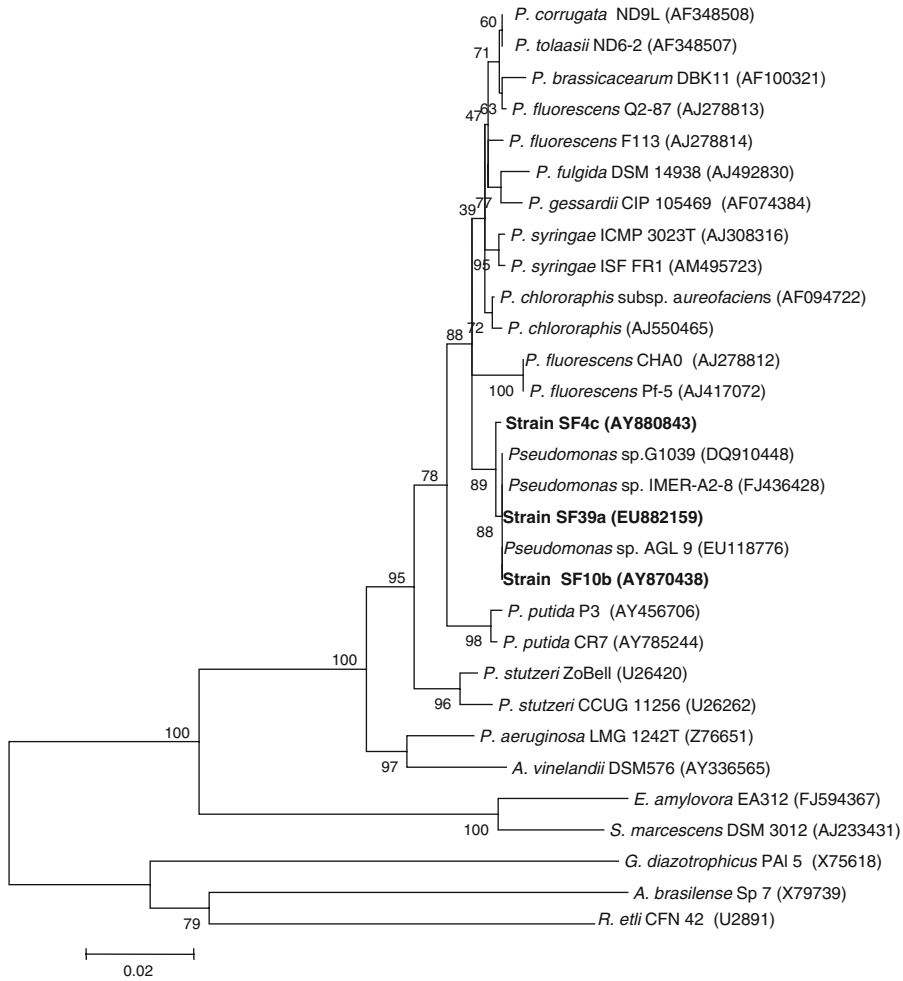

Fig. 1 Phylogenetic tree based on a comparison of the 16s rDNA gene sequences, showing relationships between the strains SF39a and SF10b and other closely related members of the genus *Pseudomonas*. The tree was constructed through the use the Jukes-Cantor distance and neighbor-joining methods. The *bootstraps values*, expressed as percentages of 1,000 replicates, are given at *branching points*. The *scale bars* show two substitution nucleotides per 100 nucleotides. The database accession numbers are indicated after the bacterial names

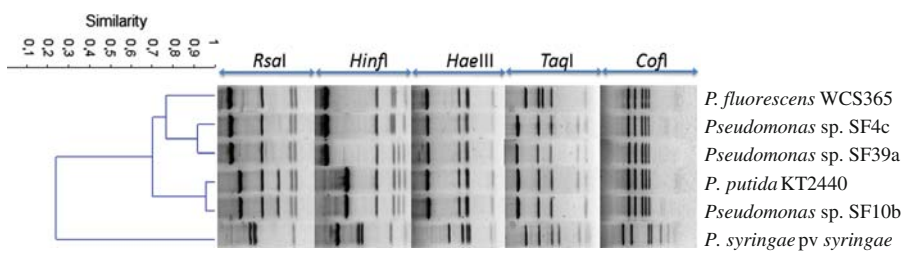

Fig. 2 Agarose gel–generated ARDRA dendrogram illustrating the relationship between native strains and reference strains of *Pseudomonas*. ARDRA-banding patterns were obtained by independent digestion of the amplified 16S rDNA with *Rsa*I, *Hae*III, *Taq*I, *Hin*fI and *Cfo*I endonucleases. A dendrogram was constructed by means UPGMA-clustering analysis based on the Jaccard similarity coefficient

Table 1 Viability of *Pseudomonas* sp. SF4c and *Pseudomonas* sp. SF10b (wild-type) and their spontaneous rifampicin-resistant mutants (*Pseudomonas* sp. SF4cR and *Pseudomonas* sp.10bR, respectively) on rich and minimal media

Strain	Number of viable cells (cfu $\times$ 10^8 ml^{-1}) on LB (pure culture)	Number of viable cells (cfu $\times$ 10^8 ml^{-1}) on GMM (pure culture)	Number of viable cells (cfu $\times$ 10^8 ml^{-1}) on LB (mixed culture)[b]	Number of viable cells (cfu $\times$ 10^8 ml^{-1}) on GMM (mixed culture)[b]
SF4c	2.20 ± 0.29	6.32 ± 0.27	1.10 ± 0.22	2.29 ± 0.38
SF4cR[a]	3.10 ± 0.34	5.10 ± 0.57	0.98 ± 0.15	2.05 ± 0.30
SF10b	4.12 ± 0.61	1.39 ± 0.09	1.08 ± 0.04	0.77 ± 0.03
SF10bR[a]	4.08 ± 0.39	1.30 ± 0.09	1.03 ± 0.04	0.61 ± 0.03

Data represent the means ± the standard error from 3 independent experiments (with 3–4 replicates in each experiment), and were analyzed by the "*t*"-test at the $P < 0.05$, without finding significant differences

[a] Viability of SF4cR or SF10bR on medium with rifampicin (100 μg ml^{-1})

[b] The number of wild-type was calculated by subtracting the number of antibiotic-resistant cells from the total number

soil (initial density) to $\log_{10}$ 4.13 cfu g^{-1} of soil after 15 days and then to $\log_{10}$ 3.77 at 30 days, thereafter remaining at these values throughout the rest of the 60 days. In bulk soil, the number of cells fell below the detection limit (Fig. 3b).

By contrast, the viable-cell number of SF4cR strain was higher in non-fertilized rhizosphere soil than in the fertilized one during the first 30 days, but at day 60 the cells density was similar under both conditions. In the non-fertilized bulk soil, the bacterial density gradually declined from $\log_{10}$ 6 cfu g^{-1} of soil (initial density) to $\log_{10}$ 4.08 at 60 days (Fig. 3c).

In non-fertilized rhizosphere soil, the population density of the SF10bR strain first decreased from $\log_{10}$ 6 cfu g^{-1} of soil (initial density) to $\log_{10}$

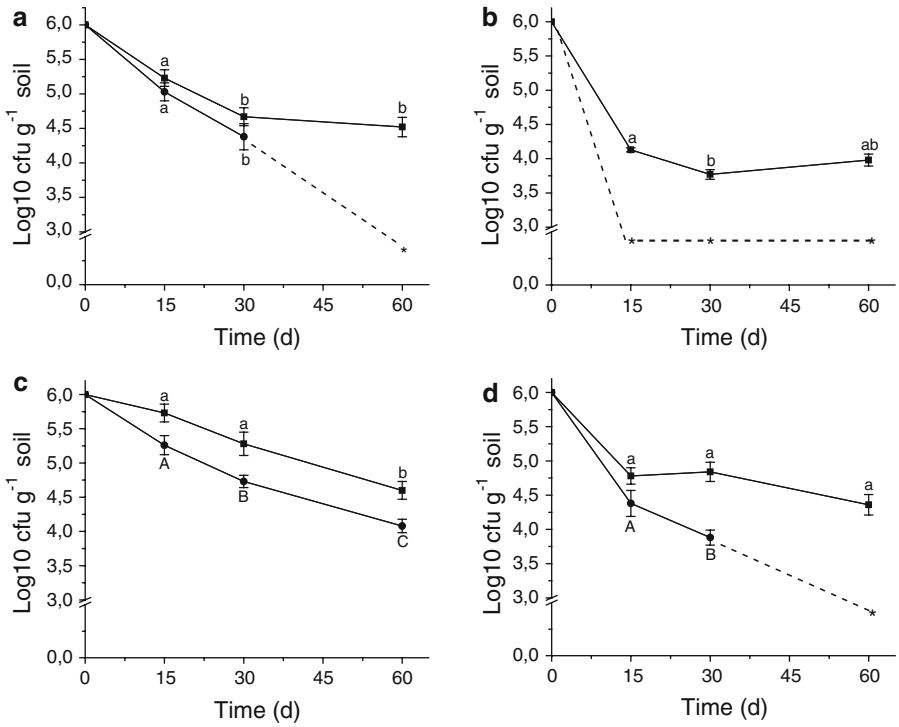

Fig. 3 Survival of *Pseudomonas* sp. SF4c (**a**, **c**) and *Pseudomonas* sp. SF10b (**b**, **d**) in the rhizosphere of wheat (*square*) and bulk soil (*circle*). **a**, **b** fertilized soil; **c**, **d** non-fertilized soil. *Dotted line* indicating viable cell number is below the detection limit. Each *point* represents the means ± SE of 3 experiments with 5–6 samples per experiments. *Different letters* indicates a significant difference ($P \leq 0.05$)

4.78 cfu g^{-1} of soil in the first 15 days, but then remained at these values during the subsequent 60 days. In the bulk soil, the number of viable cells declined to about $\log_{10}$ 4.38 at day 15, and after day 30 cfu dropped below detection limit (Fig. 3d).

As a control, the number of viable cells was determined in an uninoculated plant. Antibiotic resistant bacteria were not detected on the wheat plants from the control pots.

Growth promotion in tomato

Pseudomonas sp. SF4c and *Pseudomonas* sp. SF10b are able to promote wheat growth under greenhouse conditions (Fischer et al. 2007). We therefore evaluated here the possibility of PGPR effects on the tomato. Plants inoculated with SF10b or SF4c showed a significant increase in root and shoot dry weight and in neck diameter compared to control plants (Table 2).

Antagonistic activity of native *Pseudomonas* against phytopathogenic fungi

The antagonistic activity of native *Pseudomonas* against pathogenic strains of *Fusarium*, *Sclerotinia* and *Rhizoctonia* was tested in vitro on TSA and PDA media. *Pseudomonas* sp. SF4c was able to inhibit the mycelial growth of *S. minor* on TSA medium. By contrast, although no such adverse effect on hyphal growth occurred on PDA medium with this fungus, nevertheless sclerotia formation (indicating resistance structures) was not observed in the vicinity of the bacteria. *Pseudomonas* sp. SF10b produced a slight growth inhibition of *S. minor* on TSA but not on PDA. Both strains (SF4c and SF10b) exhibited antifungal activity against *Rhizoctonia solani* on PDA medium, but not on TSA, though they did inhibit the mycelium growth of *S. sclerotiorum* to some degree on this latter medium (Fig. 4). Neither of the strains, however, had antifungal activity against *F. verticilloides*, *F. solani*, *F. graminearum*, *F. proliferatum* on either media.

For a further characterization of this antagonistic activity, the potential of these strains to produce hydrolytic enzymes and secondary metabolites was studied, finding that none of the isolates produced

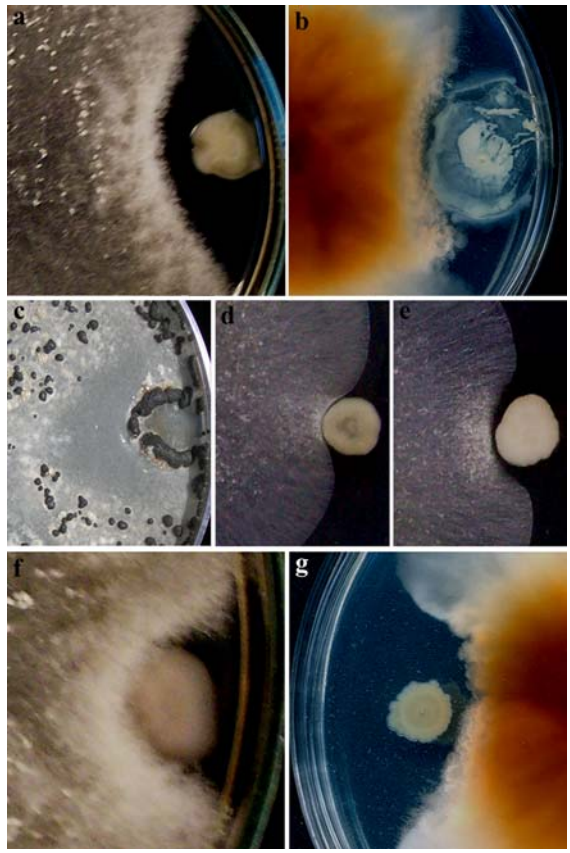

Fig. 4 Antagonist activity of SF4c (**a**–**d**) and SF10b (**e**–**g**) against phytopathogenic fungi. *Sclerotinia minor* on TSA (**a** and **f**) and PDA media (**c**); *Rhizoctonia solani* on PDA media (**b** and **g**); *Sclerotinia sclerotiorum* on TSA media (**d**–**e**)

Table 2 Effect of *Pseudomonas* sp. SF4c and *Pseudomonas* sp. SF10b on the biomass of tomatoes

Parameters	Root weight (mg)	Shoot weight (mg)	Shoot diameter (mm)
Control	29.13 ± 0.66 a	149.10 ± 4.50 a	2.31 ± 0.034 a
SF10b	34.82 ± 1.08 b	177.14 ± 5.63 b	2.52 ± 0.046 b
SF4c	36.62 ± 0.91 b	206.26 ± 6.55 c	2.64 ± 0.040 c

The data represent the means ± the standard error from 2 independent experiments $n = 25$. Different letters indicate a significant difference ($P \leq 0.05$)

chitinase nor β-glucanase. However, protease production was shown for SF4c while cellulase activity was observed in SF10b.

Svercel et al. (2007) reported the development of primers directed against sequences in the *hcnBC* genes that allow the finding of HCN-producing *Pseudomonas* spp. In doing this, a fragment of 570 bp was observed in both SF4c strain and the reference *P. fluorescens* CHA0, indicating the presence of *hcnBC* genes involved in the biosynthesis of HCN.

On the other hand, antibiotics DAPG, PCA, and PRN have been demonstrated to be responsible for the antifungal activity of many *Pseudomonas* strains. For this reason, we screened *Pseudomonas* sp. SF4c and *Pseudomonas* sp. SF10b for the presence of the biosynthetic operons encoding the tree antibiotics by PCR using specific primers (Raaijmakers et al. 1997; De Souza and Raaijmakers 2003). A fragment of the expected size for each one of these antibiotics was observed in the reference strains of *Pseudomonas* that produce DAPG, PCA or PRN; but no such fragments were amplified from the DNA of any of the native strains.

Discussion

Native strains from wheat rhizosphere with PGPR traits have been previously isolated in our laboratory. One such strain was identified as belonging to the *Pseudomonas* genus and was designated *Pseudomonas* sp. SF4c (Fischer et al. 2007). In this present report, when PCR amplification was performed with *Pseudomonas*-specific primers, two other strains (SF10b and SF39a) were identified as members of this genus. These results were confirmed by means of the sequencing of 16S rDNA.

Survival and physiologic response to soil conditions of strains released into the field constitute important information to gain a better insight into the bacteria′s behaviour under natural conditions. Thus, microcosm experiments are a transition between controlled laboratory experiments and field ones, with fluctuating soil and climatic conditions (van Overbeek et al. 1997). The use of bacteria with antibiotic-resistance markers is a simple and rapid method to facilitate the detection and quantification of bacteria introduced into both the soil and the rhizosphere of a plant, without interfering with fitness of the organism. For this reason, spontaneous antibiotic-resistant mutants have been widely used in ecological studies (Nautiyal 1997; Egamberdiyeva and Höflich 2003; Kumar et al. 2007). To monitor the survival of *Pseudomonas* sp. SF4c and *Pseudomonas* sp. SF10b in soil, we first obtained spontaneous rifampicin-resistant mutants derived from SF4c and SF10b. These strains, designed SF4cR and SF10bR, were as competitive for growth as their parental strains and were therefore used to monitor survival and persistence of bacteria in the wheat rhizosphere and the bulk soil. The number of viable cells of strains SF4cR and SF10bR declined during the first days. They then exhibited a general tendency to become established in the wheat rhizosphere at an appropriate cell density in both soil types (fertilizer and non-fertilizer). Numerous studies have demonstrated, for a wide variety of introduced bacteria into soil, that in general, bacterial populations decline following introduction into a natural soil, owing to the hostility of adverse abiotic and biotic influences within the soil (van Veen et al. 1997). Van Elsas and van Overbeek (1993) reported population-decline rates of fluorescent pseudomonads in various soil types as being from 0.2 to 1.1 log units per 10-days period, although larger rates have also been reported (Turnbull et al. 2001). Kumar et al. (2007) found that *P. corrugata* viability counts were reduced from the initial $\log_{10}$ 11.26 cfu g^{-1} soil to $\log_{10}$ 8.07 cfu g^{-1} at 28 days in the maize rhizosphere, after which time the population tally remained stable.

Survival of the SF4cR and SF10bR strains was superior in the rhizosphere than in the bulk soil. Similar results have been previously observed with different strains of *Pseudomonas* (Molina et al. 2000; Johansen et al. 2005).

Beneficial effects of plant-growth-promoting rhizobacteria, and particularly those of the genus *Pseudomonas*, in enhancing growth and improving plant health are well known. Previous studies with the strains SF4c and SF10b had demonstrated that both are able to promote wheat growth under greenhouse conditions probably because they presented some PGPR traits such as siderophore or IAA production or solubilization of phosphate (Fischer et al. 2007). In this work, we analyzed the ability of these strains to facility growth in an additional plant host. In this sense, the inoculation of tomatoes with SF4c and SF10b, under greenhouse conditions, had a positive

effect on the biomass. We can therefore conclude that both strains were PGPRs in either wheat or tomato.

Finally, antagonist activity of the strains SF4c and SF10b against phytopathogenic fungi was evaluated in vitro. Members of the genera *Sclerotinia*, *Fusarium* and *Rhizoctonia* are pathogens that infect a wide range of plants including several crops, vegetables, and fruit and produce significant economic losses (Edwards 2004; Purdy 1979; Sneh et al. 1991). *Pseudomonas* sp. SF4c and *Pseudomonas* sp. SF10b inhibited the mycelial growth (or their resistance structures) in some of the phytopathogenic fungi tested. Variation in the antagonism of the strains SF4c and SF10b against fungi was observed on different solid media. These results are in agreement with McSpadden et al. (2005), who found that medium composition influenced the pathogen-inhibition activities expressed by different *phlD*+ genotypes of *Pseudomonas*. For example, the A genotype, but not the D and the S, inhibited pytopathogenic fungi on media derived from soybean and corn (TSA and CMA). The A genotype was thus considered to be more likely to express biocontrol activities in the rhizosphere of corn and, especially on soybean plants, than would be the D and the S genotypes. This finding indicated that the biocontrol activities of these pseudomonads could likely vary among different crops (McSpadden et al. 2005).

With the intention of further characterizing the antagonistic potential of *Pseudomonas* sp. SF4c and *Pseudomonas* sp. SF10b as biocontrol agents, we screened them for the presence of genes enconding the antibiotics DAP, PCA, PRN or HCN by PCR with specific primers. The production of extracellular enzymes was also determined in these strains. Neither of them proved to be positive in the PCR-reaction for DAP, PCA and PRN, thus indicating that they are not producers of these antibiotics. On the contrary, the essential genes for HCN biosynthesis were detected in SF4c; both strains produced some extracellular lytic enzymes and we have previously demonstrated that they also produce siderophores.

In a similar way, Costa et al. (2006) demonstrated that among thirteen native *Pseudomonas* spp. displaying antagonistic activity against phytopathogenic fungi, only three were positive for the detection of antibiotic-encoding genes by PCR (2 phlD+ and 1 prnD+) but the majority produced siderophores and protease. Moreover, Berg et al. (2005) found that eighteen out of twenty antagonistic isolates expressed proteolytic activity and 17 isolates expressed cellulytic activity and siderophore production, whereas only three isolates showed chitinolytic activity. In addition, *Lysobacter enzymogenes* C3 is a bacterial biological control agent that exhibits antagonism against multiple fungal pathogens and its antifungal activity has been attributed in part to production of lytic enzymes (Li et al. 2008).

In conclusion, results presented in this paper demonstrate that *Pseudomonas* sp. SF4c and *Pseudomonas* sp. SF10b could be good candidates to develop plant inoculants. These native strains became established in the wheat rhizosphere, competed satisfactorily with the indigenous microflora, and persisted in adequate viable-cell numbers in the rhizosphere. Moreover, both strains inhibited of the growth of certain phytopathogenic fungi, though the antagonist activity depended on which medium the test was performed.

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