Journal of Microbiological Methods 48 (2002) 69–86

Journal of Microbiological Methods

www.elsevier.com/locate/jmicmeth

A polyphasic approach for studying the interaction between *Ralstonia solanacearum* and potential control agents in the tomato phytosphere

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Received 4 July 2001; received in revised form 3 September 2001; accepted 5 September 2001

Abstract

Ralstonia solanacearum biovar 2, the causative agent of brown rot in potato, has been responsible for large crop losses in Northwest Europe during the last decade. Knowledge on the ecological behaviour of *R. solanacearum* and its antagonists is required to develop sound procedures for its control and eradication in infested fields. A polyphasic approach was used to study the invasion of plants by a selected *R. solanacearum* biovar 2 strain, denoted 1609, either or not in combination with the antagonistic strains *Pseudomonas corrugata* IDV1 and *P. fluorescens* UA5-40. Thus, this study combined plating (spread and drop plate methods), reporter gene technology (*gfp* mutants) and serological (imunofluorescence colony staining [IFC]) and molecular techniques (fluorescent in situ hybridization [FISH], PCR with *R. solanacearum* specific primers and PCR–DGGE on plant DNA extracts). The behaviour of *R. solanacearum* 1609 and the two control strains was studied in bulk and (tomato) rhizosphere soil and the rhizoplane and stems of tomato plants. The results showed that an interaction between the pathogen and the control strains at the root surface was likely. In particular, *R. solanacearum* 1609 CFU numbers were significantly reduced on tomato roots treated with *P. corrugata* IDV1(*chr::gfp*1) cells as compared to those on untreated roots. Concomitant with the presence of *P. corrugata* IDV1(*chr::gfp*1), plant invasion by the pathogen was hampered, but not abolished. PCR–DGGE analyses of the tomato rhizoplane supported the evidence for antagonistic activity against the pathogen; as only weak *R. solanacearum* 1609 specific bands were detected in profiles derived from mixed systems versus strong bands in profiles from systems containing only the pathogen. Using FISH, a difference in root colonization was demonstrated between the pathogen and one of the two antagonists, i.e. *P. corrugata* IDV1(*chr::gfp*1); *R. solanacearum* strain 1609 was clearly detected in the vascular cylinder of tomato plants, whereas strain IDV1 was absent. *R. solanacearum* 1609 cells were also detected in stems of plants that had developed in soils treated with this strain, even in cases in which disease symptoms were absent, indicating the occurrence of symptomless infection. In contrast, strain 1609 cells were not found in stems of several plants treated with either one of the two antagonists. The polyphasic analysis is valuable for testing antagonistic strains for approval as biocontrol agents in agricultural practice. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: *Ralstonia solanacearum*; Tomato phytosphere; *Pseudomonas corrugata*; *Pseudomonas fluorescens*

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PII: S0167-7012(01)00347-5

1. Introduction

In the past decade, brownrot disease re-appeared in Dutch potato fields after a long period of absence. A considerable amount of farmland used for starch potato production, was infested by the pathogen *Ralstonia solanacearum* biovar 2 (race 3), resulting in major economic losses (Van Elsas et al., 2000; Wenneker et al., 1999).

Recent studies have addressed the survival of *R. solanacearum* biovar 2 (strain 1609) in soil and water from these regions (Van Elsas et al., 2000; 2001). The pathogen revealed the capacity to survive in these habitats for considerable periods of time (Graham et al., 1979; Van Elsas et al., 2000; 2001). Temperature strongly influenced the survival of *R. solanacearum*, as optimal survival, in particular in water systems, was demonstrated at physiologically favorable temperatures (Van Elsas et al., 2001) and reduction of *R. solanacearum* populations was observed at low temperatures (Van Elsas et al., 2000; 2001). Evidence for the occurrence of viable-but-non-culturable (VBNC) cells of the pathogen was obtained in soil and water microcosms (Van Elsas et al., 2000; 2001). The observation of these VBNC cells, but also the appearance of symptomless infections of the pathogen in, for instance, *Capsicum annuum* (Abdullah and Rahman, 1998), indicate the limitations of cultivation-based procedures and observation of plant disease symptoms (Graham and Lloyd, 1975) as sole methods for the detection of *R. solanacearum*.

Strategies for the biological control of *R. solanacearum* are still in a developmental stage (Trigalet et al., 1998; Smith et al., 1998). An important criterion for the selection of biological control strains is optimal survival at the roots of host plants, but even strains selected as optimal survivors may fail when used as biocontrol agents. It was, therefore, realized that a thorough understanding of the ecological relationships between plant, pathogen and biocontrol strain is required, which can only be achieved by combining a suite of robust monitoring methods that provide different perspectives of these organisms in the same habitat.

R. solanacearum is routinely detected, by plant protection services, via plating onto the semi-selective medium SMSA (culturable count of presumptive *R. solanacearum*; Engelbrecht, 1994; Elphinstone et al., 1996) and immunofluorescence (IF, cell count; Janse, 1988; Elphinstone et al., 1996). In addition, immunofluorescence colony staining (IFC; Van Vuurde and Van der Wolf, 1995; Van der Wolf et al., 1998; Van Elsas et al., 2000) has been proposed as an adequate method for ecological monitoring, as it specifically identified and enumerated the culturable cells of *R. solanacearum*.

For the specific detection of *R. solanacearum* at the DNA level, primer systems have been developed by different groups (Seal et al., 1993; Boudazin et al., 1999). The system recommended by Boudazin et al. (1999) is apparently superior for the detection of *R. solanacearum* division 2, to which biovar 2 belongs, but cross-reactions with indigenous soil organisms can still occur (Van Elsas et al., 2000).

PCR-denaturing gradient gel electrophoresis (DGGE) based on 16S ribosomal RNA genes allows molecular fingerprints to be made of microbial communities in bulk soil (Akkermans et al., 1995; Felske et al., 1998; Rosado et al., 1998; Gelsomino et al., 1999; Duarte et al., 2001), rhizosphere soil (Duineveld et al., 1998, 2001) and in plant tissue (Garbeva et al., 2001). The application of this fingerprinting method to bacterial assemblages in the rhizosphere represents an elegant approach to the study of pathogen–antagonist relationships near or inside plants. A major advantage of this technique is that it is able to provide evidence for putative microbial interactions in the same biotope.

Detection of single cells or microcolonies in natural habitats can be achieved using in situ hybridization on the basis of 16S ribosomal RNA-directed oligonucleotides (Hahn et al., 1993; Amann et al., 1995; Amann, 1998). Two oligonucleotide probes that specifically targeted the 23S ribosomal RNA of *R. solanacearum*, RSOLA and RSOLB, have recently been described by Wullings et al. (1998). Although in particular probe RSOLB was shown to specifically detect *R. solanacearum*, the quality of detection may even be improved by combining in situ hybridization with immunofluorescence, as was demonstrated with *Clavibacter michiganensis* spp. *sepedonicus* in plants (Li et al., 1997).

The use of reporter or marker genes such as *lux* (Fravel et al., 1990; De Weger et al., 1997), *luc* (Jansson et al., 2000) or *gfp* (Leff and Leff, 1996; Bloemberg et al., 1997; Normander et al., 1999; Tom-

bolini et al., 1999; Cassidy et al., 2000) is useful for the in situ detection of microcolonies and individual cells associated with plants. In addition, the marker genes can be used in plating assays for the detection of culturable cells. Using different markers (e.g. *lux* and *gfp*), colonization of the vascular system of host plants by different phytopathogens has been recently demonstrated (Bogs et al., 1998; Hikichi et al., 1998).

In this study, the interplay between two potential biocontrol agents, i.e. *Pseudomonas corrugata* IDV1 and *P. fluorescens* UA5-40 (Zablotowicz et al., 1995), and *R. solanacearum* bv 2 strain 1609 in soil/plant microcosms was assessed using a combination of the aforementioned techniques. Both potential antagonists were labeled with *gfp*, whereas the wild-type strain 1609 was used. The assessment of the effect of the antagonists on the invasion of tomato plants by *R. solanacearum* was a major objective.

2. Materials and methods

2.1. Bacterial strains, insertion of gfp and growth conditions

R. solanacearum bv 2 (race 3), strain 1609, has been isolated by the Dutch Plant Protection service from infected potato tubers (cv Bartina) (Van Elsas et al., 2000). The isolate was regularly tested for its aggressiveness by injection into tomato plants. After incubation under the appropriate conditions (Graham and Lloyd, 1975), all treated plants revealed symptoms typical for brownrot, i.e. severe wilting, growth reduction and overproduction of anthocyanins, within 1–2 weeks.

P. corrugata strain IDV1 was isolated from water surrounding the roots of *R. solanacearum*-infested bittersweet (*Solanum dulcamara*) plants. Clearance zones near colonies of strain IDV1 on ‘‘indicator’’ plates (prepared by mixing one-tenth strength Trypticase Soy Broth Agar ($0.1 \times$ TSBA: TSB [Becton Dickinson, MD, USA], 3 g; agar, 15 g; water 1 l) with *R. solanacearum* 1609 cells) indicated antagonistic activity of IDV1 against the pathogen. Strain IDV1 was identified by BIOLOG™ plates as *P. corrugata* (level of similarity 0.686). *P. fluorescens* UA5-40 was kindly provided by Dr. R. Zablotowicz (Zablotowicz et al., 1995).

Derivatives of strains IDV1 and UA5-40, carrying *gfp* insertions, were obtained via electroporation (Unge et al., 1998). The pUT*gfp* suicide vector (pUT vector loaded with mini-Tn5 *npt*II-*gfp*; kindly provided by Dr. J. Jansson, Stockholm University, Sweden) was used to mark strain IDV1, whereas the pJB29-*gfp*-mut3b vector (Møller et al., 1998) was used for strain UA5-40. Transformants obtained by selection for kanamycin resistance were tested for *gfp* gene expression by low magnification epifluorescence microcopy. Mutants with the highest expression were isolated; these were denoted IDV1(*chr::gfp*1) and UA5-40G2, respectively. All strains were kept at -80 °C in 20% glycerol.

Strains 1609, IDV1 and IDV1(*chr::gfp*1) were grown in $0.1 \times$ TSB and strains UA5-40 and UA5-40G2 in Luria–Bertani broth (LB: tryptone, 10 g; yeast extract, 5 g; NaCl, 5 g; H_2O, 1 l; pH 7.2) with (*gfp*-marked strains) or without 50 μg/ml of kanamycin. Cultures were incubated with shaking at 27 °C. Cells in end-logarithmic phase were harvested by centrifugation and washed three times in sterile demineralized water.

2.2. Soil treatment and experimental set up

Fresh potting soil (Trio potting soil 17, Klasman Benelux, The Netherlands; characteristics: organic matter 68%, pH 5.9) was amended, either with water (untreated soil) or with washed bacterial cell suspensions, establishing a final water content of 270% (corresponding to about pF 1.5). Soil portions (100 g) to be inoculated with pathogen/antagonist combinations were first treated with *R. solanacearum* 1609 followed by addition of either of the two antagonists. Final densities of inoculant strains were set at Log 7.7 (*R. solanacearum* 1609) or Log 7.5 (strains IDV1-(*chr::gfp*1) and UA5-40G2) CFU per gram of dry soil. Three-week-old tomato plants (*Lycopersicon esculentum* Mill. cv Moneymaker) were treated and planted into inoculated soils as follows (Table 1): (i) plants remained undamaged and were transferred to unamended (control) soil or soils with strain 1609 with or without strains IDV1(*chr::gfp*1) or UA5-40G2 (soil treatment), (ii) roots were damaged by cutting three side branches, prior to planting into soil containing strain 1609 (damaged root treatment), (iii) roots were dipped in suspensions of strains IDV1(*chr::gfp*1) or UA5-40G2 (density Log 8.7 CFU/ml), prior to planting into soils with the

Table 1
Experimental set-up and number of diseased or infected plants after 8 and 14 days

Treatment[a]	Number of plants sampled at day:		Number of wilted[b] and infected[c] plants after 8 and 14 days, respectively	
	8	14	Wilted	Infected
(a) Untreated soil, intact roots	1	1	0	0
(b) *R. solanacearum* 1609 in soil, intact roots	3	2	3	5
(c) *R. solanacearum* 1609 in soil, damaged roots	2	3	5	5
(d) *P. corrugata* IDV1(*chr::gfp*1) in soil, intact roots	2	2	0	ND[d]
(e) *P. fluorescens* UA5-40G2 in soil, intact roots	2	2	0	ND
(f) As d, roots dipped in suspension of *P. corrugata* IDV1(*chr::gfp*1)	2	2	0	ND
(g) As e, roots dipped in suspension of *P. fluorescens* UA5-40G2	2	2	0	ND
(h) *R. solanacearum* 1609 and *P. corrugata* IDV1(*chr::gfp*1) in soil, intact roots	3	3	0	6
(i) *R. solanacearum* 1609 and *P. fluorescens* UA5-40G2 in soil, intact roots	3	3	3	6
(j) As h and roots dipped in suspension of *P. corrugata* IDV1(*chr::gfp*1)	3	3	0	2
(k) As i and roots dipped in suspension of *P. fluorescens* UA5-40G2	3	3	0	5

[a] Treatment; tomato (*L. esculentum* var. moneymaker) plants were planted in potting soil with the following treatments: soil remained uninoculated (a) or was treated with log 7.7 *R. solanacearum* 1609 CFU/g of dry soil (treatments b, c, h–k); roots of plants remained undamaged (treatments a, b and d–k) or side branches were sliced from the roots prior to planting (c); soils were not further inoculated (a–c) or received log 7.5 *P. corrugata* IDV1(*chr::gfp*1) or *P. putida* UA5-40G2CFU/g of dry soil (d–k); roots remained untreated (a–e, h, i) or were dipped in suspension of log 8.7 *P. corrugata* IDV1(*chr::gfp*1) or *P. putida* UA5-40G2 CFU/ml prior to planting in soil (f, g, j, k).

[b] Number of plants showing symptoms of brown rot.

[c] Number of plants giving a positive signal both by PCR by using primers D2/B (Boudazin et al., 1999) and IFC by using an FITC-conjugated antiserum raised against *R. solanacearum* bv 2.

[d] ND; not determined.

same strain and with or without strain 1609 (root dipping treatment).

Plant–soil microcosms were incubated for 8 or 14 days under a light/dark regime (26 °C 16 h/21 °C 8 h) at air humidity of 70%. Two or three pots from each treatment were destructively sampled at each sampling time (8 and 14 days), whereas one control pot was analysed at both times.

2.3. Sampling

At each sampling, plants were carefully removed from the soil, after which 10-g samples of the remaining soil (bulk soil samples) were transferred to Erlenmeyer flasks with 95 ml of sterile 0.1% sodium pyrophosphate solution (NaPPi), and treated according to Postma et al. (1988). Roots with adhering soil were cut from the plants and divided in two portions. From one portion, material was transferred to fresh NaPPi flasks which were shaken (yielding rhizosphere soil suspensions). Following shaking, the roots were carefully removed from the suspensions, washed in sterile demineralized water and divided in two subportions (one for analysis of the rhizoplane by cultivation-based methods and the other one for fluorescent in situ hybridization [FISH] analysis). The second portion of roots with soil was carefully rinsed in sterile demineralized water; roots were then sliced in 1-cm pieces from the stem base downwards, resulting in about seven pieces per plant, which ranged between 0.2 and 1 g fresh weight (for DNA extraction).

Stems of the individual plants were cut off, divided in 0.5–1 cm pieces, and separated in two portions ranging in weight between 0.2 and 1 g. These stem part portions were surface-sterilized using 70% ethanol.

Both stem and root parts (see above) were treated as follows: (1) One portion each of root (rhizoplane) and

stem parts was homogenized in 3 ml of sodium phosphate buffer (NaP: $Na_2HPO_4 \cdot H_2O$, 19.9 g; $NaH_2PO_4 \cdot 2H_2O$, 1.27 g; H_2O, 1 l: pH 8) (for CFU recovery and DNA extraction), (2) a second portion of roots was transferred to a 3% (wt/v) solution of paraformaldehyde dissolved in phosphate-buffered saline (PBS; $Na_2HPO_4 \cdot H_2O$, 13.8 g; $NaH_2PO_4 \cdot H_2O$, 3.5 g; NaCl, 8.5 g, 1 l; pH 7.4) for fixation of bacterial cells followed by FISH analysis (see later).

2.4. Cultivation-based assessments

Replicate aliquots from bulk and rhizosphere soil suspensions and root (rhizoplane) or stem homogenates were serially diluted in 0.1% sodium pyrophosphate (NaPPi) solution, and both spotted (drop plate method; Cassidy et al., 2000) and spread plated (100 μl) onto LBA plates, either amended with 100 μg/ml of cycloheximide (Ch) alone (unselective counts; drop plate method), or in combination with 50 μg/ml of kanamycin (Km) (selective counts). These soil suspensions and homogenates were also transferred to 24-well microtiterplates and treated according to Van der Wolf et al. (1998) (IFC analysis). Petri dishes and microtiterplates were incubated at 27 °C for 2 to 5 days.

R. solanacearum 1609 colonies in microtiterplate wells were stained with an fluorescein isothiocyanate (FITC)-conjugated polyclonal antiserum raised against *R. solanacearum* bv 2 (Van der Wolf et al., 1998). Immunofluorescent and *gfp*-expressing colonies (in microtiter plate [GFP–CFU] and drop plate method) were enumerated using an epifluorescence binocular (Wild M32, Heerbrugg, Switzerland) at low magnification (40 ×), whereas colonies on spread plates were counted without magnification under normal light.

2.5. DNA extraction from tomato root and stem parts, and analysis by PCR and PCR–DGGE

DNA was extracted from stem and rinsed root parts (rhizoplane) according to protocol I of Garbeva et al. (2001). DNA extracts were further purified using the Wizard® DNA clean-up system (Promega, Madison, WI, USA), establishing a final volume of 100 μl.

For detection of *R. solanacearum* 1609, DNA extracts were subjected to PCR amplification with primers D2 and B as described by Boudazin et al. (1999). For analysis of bacterial community diversity by PCR–DGGE, PCR amplification was performed on 1 μl of DNA extract (approximately 20 ng of DNA) in standard 50 μl reaction mixtures (Van Elsas and Wolters, 1995) with primers 968F (with GC-clamp; Muyzer et al., 1993) and 1401R, both directed towards 16S ribosomal DNA region V6 (Heuer and Smalla, 1997; Heuer et al., 1999). PCR amplification was performed in a PTC-200 (MJ Research, MA, USA) thermocycler.

PCR products were analysed in standard ethidium bromide stained agarose gels (Sambrook et al., 1989) or in 6% acrylamide gels containing a denaturing gradient of 45–65% of urea/formamide (DGGE) under conditions described by Rosado et al. (1998). Bands in DGGE gels were visualized by silver staining (Bio-Rad Laboratories, CA, USA) according to the manufacturer's protocol.

2.6. Fluorescent in situ hybridization

Tomato stem pieces were treated with 3% paraformaldehyde solution (4 h at 4 °C), washed in PBS, and stepwise dehydrated in progressively increasing ethanol concentrations (Wullings et al., 1998). Cell staining was performed with the bacterial probe EUB338 (Amann et al., 1995) labeled with FITC, and with the *R. solanacearum*-specific probe RSOLB (Wullings et al., 1998), labeled with Cy3. Hybridization of the dehydrated stem pieces was performed according to Wullings et al. (1998). Stained cells in stem pieces were detected by epifluorescence microscopy (Zeiss Axioskop, Göttingen, Germany), at magnification of 100 × , and photographed with a Coolsnap digital camera (RS photometrics, Ca, USA).

2.7. Statistical analyses

Statistical analyses were performed on duplicate or triplicate samples. Comparisons were made by analysis of variance (Genstat 5, release 4.1, PC/Windows 98 version, Rothamsted Experimental Station, Harpenden, UK), and standard errors of difference were calculated. Values were considered to be significantly different at a 95% (or higher) confidence level. Variable values (CFU, GFP–CFU and IFC numbers) were log transformed and the following factors were considered: soil and plant treatments, sampling time

and plating method (selective/unselective drop and spread plate and GFP–CFU methods).

3. Results

3.1. Plant health parameters

All 18 tomato plants grown in untreated soils and in soils inoculated only with strain *P. corrugata* IDV1 (*chr::gfp*1) or *P. fluorescens* UA5-40G2 remained healthy after 8 and 14 days. In contrast, in 11 (out of 34) plants grown in *R. solanacearum* 1609-infested soil, disease symptoms were observed within 14 days of incubation (Table 1); in soil containing only *R. solanacearum* 1609, three (out of five) plants with intact roots showed bacterial wilt, whereas all five plants with damaged roots were diseased (Table 1 treatment b and c, respectively). In soil treated with *R. solanacearum* 1609 plus strain UA5-40G2 (Table 1, treatment i), three (out of six) tested plants showed wilting. No symptoms were observed in plants grown in soil with *R. solanacearum* 1609 when strain UA5-40G2 was added by root dipping plus soil addition (treatment k) and in soil with *R. solanacearum* 1609 to which strain IDV1(*chr::gfp*1) had been added, either or not accompanied by root dipping (treatments h and j).

3.2. Detection and dynamics of culturable populations in bulk and rhizosphere soil, and the rhizoplane, of tomato plants

3.2.1. Detection of culturable populations

The average total bacterial CFU counts in bulk and rhizosphere soils were about log 9.1 CFU/g of dry soil, whereas log 7.3 CFU/g of fresh root were found in the rhizoplane. No green fluorescence was observed in colonies obtained from untreated bulk and rhizosphere soil or from untreated plants. Also, no IFC-stainable colonies were found in these samples. The lack of background in the plant/soil systems used indicated the absence of interference with the antagonist and pathogen detection methods employed.

As expected, after staining with the *R. solanacearum* specific antiserum, bright fluorescent colonies typical for *R. solanacearum* strain 1609 (Van Elsas et al., 2000), were observed in all bulk and rhizosphere soil and rhizoplane samples obtained from systems that had originally received *R. solanacearum* strain 1609 (Table 2, treatments b, c and h–k). IFC-stainable colonies were also observed in the stems of plants grown in these treated soils (Table 1, treatments b, c, h and i), with the exception of several plants of which the roots had been dipped in suspensions of strain IDV1(*chr::gfp*1) (four out of six plants) or strain UA5-40G2 (five out of six plants) (treatments j and k).

In the drop and spread plate as well as the IFC assessments, the fluorescence of IDV1(*chr::gfp*1) colonies was slightly lower than that of strain UA5-40G2 colonies (Fig. 1), which offered a second criterion for strain identification during detection. In IFC of samples from systems with mixed inoculants (treatments h–k), the *gfp*-expressing colonies could be easily distinguished from those of *R. solanacearum* 1609 by their larger colony size (Fig. 1). In addition, *R. solanacearum* revealed a very distinctive, disk-shaped, colony morphology, which was different from the morphologies of the control strains. This, thus, offered the possibility of side-by-side detection of the biocontrol and pathogen strains.

Using the drop and spread plate and GFP–CFU methods, colonies emitting green fluorescence were detected in bulk and rhizosphere soil, and rhizoplane samples from all systems that had originally received the *gfp*-marked strains (Tables 1 and 2, treatments d–k). No statistical differences were observed between the selective and unselective counts obtained with the drop plate method and the selective counts from the spread plate method (data not shown). In addition, the GFP–CFU numbers obtained in IFC were also statistically similar. Therefore, only the GFP–CFU numbers are presented in Table 2. Given the ease of detecting pathogen and antagonist in one approach, the IFC method is recommended for future studies.

3.2.2. Dynamics of culturable populations

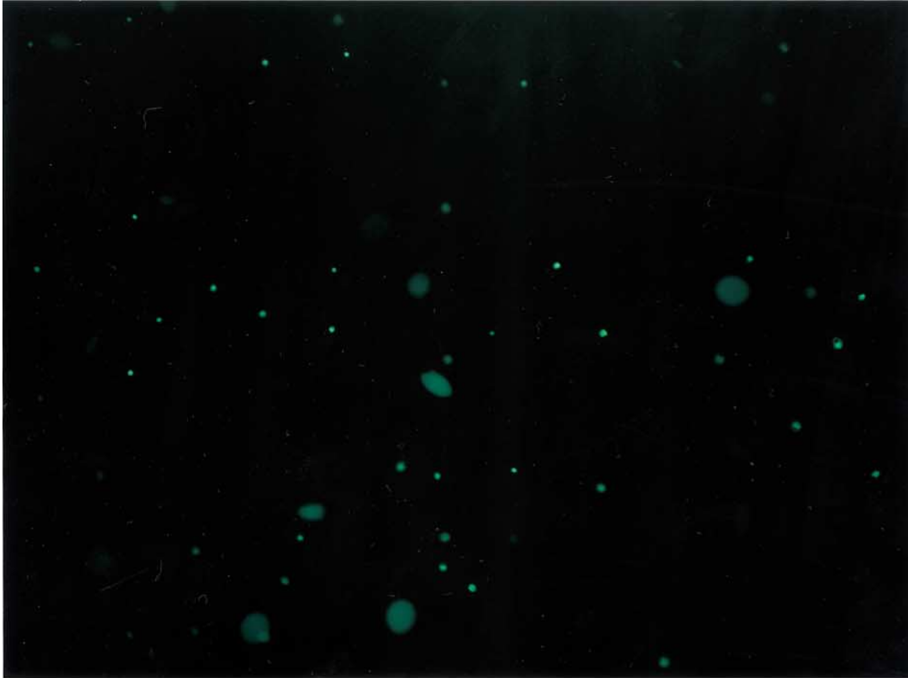
GFP–CFU numbers in bulk and rhizosphere soils and the rhizoplane in systems containing strain IDV1(*chr::gfp*1) or UA5-40G2 (Table 2, treatments d–k), ranged from log 4.58 to 6.01 (day 8, bulk soil), log 4.67 to 5.73 (day 14, bulk soil), log 5.71 to 7.38 (day 8, rhizosphere soil), log 5.74 to 6.77 (day 14, rhizosphere), log 5.38 to 7.14 (day 8, rhizoplane) and log 5.65 to 7.22 (day 14, rhizoplane) CFU per gram of dry soil or per gram of fresh root, respectively.

Table 2
Survival in soil, rhizosphere and rhizoplane of *P. putida* UA5-40G2, *P. corrugata* IDV1(*chr::gfp*1) and *R. solanacearum* 1609

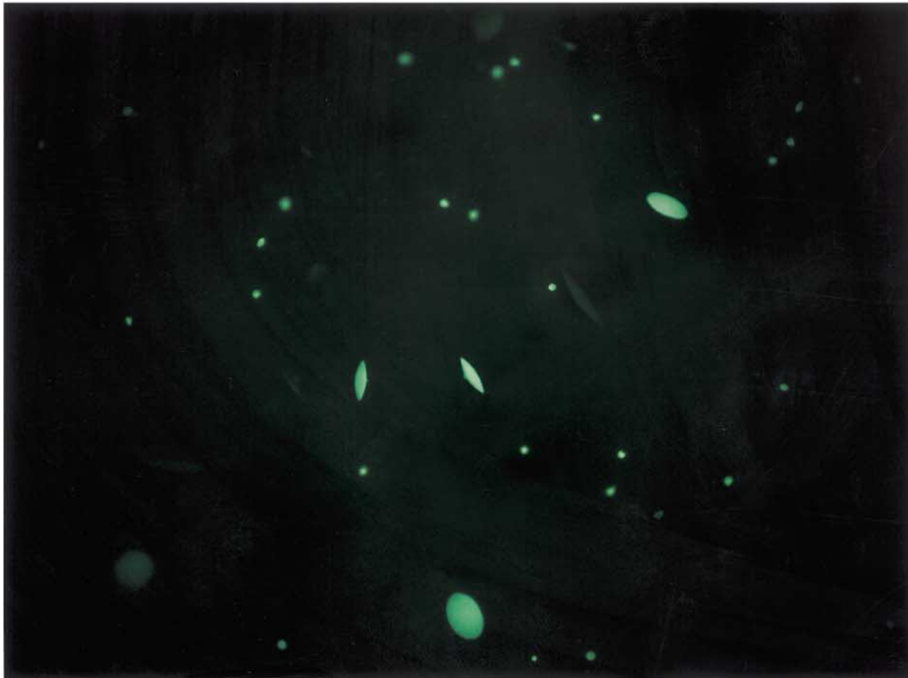
Treatment[1]	Bulk (log CFU/g of dry soil)				Rhizosphere (log CFU/g of dry soil)				Rhizoplane (log CFU/g of fresh root)			
	Biocontrol agent[2,3]		*R. solanacearum*[4]		Biocontrol agent[3]		*R. solanacearum*[3]		Biocontrol agent		*R. solanacearum*[3]	
	8 days	14 days	8 days	14 days	8 days	14 days	8 days	14 days	8 days	14 days	8 days	14 days
(a) Untreated soil	BD[5]	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD	BD
(b) *R. solanacearum* 1609 in soil, intact roots	ND[6]	ND	7.54	7.31	ND	ND	8.75^{b}	8.40^{b}	ND	ND	8.46^{b}	8.28^{b}
(c) *R. solanacearum* 1609 in soil, damaged roots	ND	ND	7.24	7.33	ND	ND	8.31^{b}	8.30^{b}	ND	ND	8.75^{b}	8.66^{b}
(d) IDV1(*chr::gfp*1) in soil, intact roots	4.58^{a}	4.67^{a}	ND	ND	6.01^{a}	6.30^{b}	ND	ND	5.38^{a}	6.45^{a}	ND	ND
(e) UA5-40G2 in soil, intact roots	4.58^{a}	5.10^{a,b}	ND	ND	5.71^{a}	6.29^{b}	ND	ND	5.98^{a}	5.65^{a}	ND	ND
(f) As d, intact roots dipped in IDV1(*chr::gfp*1)	4.72^{a}	5.21^{a,b}	ND	ND	6.45^{a}	6.29^{b}	ND	ND	5.65^{a}	6.77^{a}	ND	ND
(g) As e, intact roots dipped in UA5-40G2	4.96^{a}	4.87^{a}	ND	ND	6.08^{a}	5.74^{a}	ND	ND	6.44^{a,b}	5.72^{a}	ND	ND
(h) Strains IDV1(*chr::gfp*1) and 1609 in soil	4.97^{a}	4.97^{a}	7.11	7.77	7.14^{b}	6.61^{b,c}	7.91^{b}	8.35^{b}	6.14^{a}	6.57^{a}	8.26^{b}	8.33^{b}
(i) Strains UA5-40G2 and 1609 in soil	5.57^{b}	5.08^{a,b}	7.77	7.79	6.77^{b}	6.77^{c}	8.36^{b}	8.95^{b}	5.42^{a}	7.22^{b}	8.52^{b}	8.80^{b}
(j) As h and roots dipped in IDV1(*chr::gfp*1) cells	4.94^{a}	5.73^{b}	6.95	7.21	7.38^{b}	6.25^{b}	7.29^{a}	7.15^{a}	6.96^{b}	6.50^{a}	7.49^{a}	6.59^{a}
(k) As i and roots dipped in UA5-40G2 cells	6.01^{b}	5.62^{b}	6.87	7.27	7.19^{b}	6.34^{b}	7.01^{a}	7.44^{a}	7.14^{b}	6.75^{a}	7.41^{a}	8.42^{b}

[1] Treatment of the soil, see legend of Table 1.
[2] Biocontrol agent; *P. putida* UA5-40G2 or *P. corrugata* IDV1(*chr::gfp*1) GFP–CFU numbers determined on selective plates.
[3] a, b, c; significantly different ($P<0.05$). Difference in log CFU numbers were determined by analysis of variance ($a<b<c$).
[4] *R. solanacearum*; *R. solanacearum* 1609 CFU numbers determined by immunofluorescence colony counts.
[5] BD; below detection; <log 3 CFU per gram of dry soil or gram of fresh root.
[6] ND; not determined.

(a)



(b)



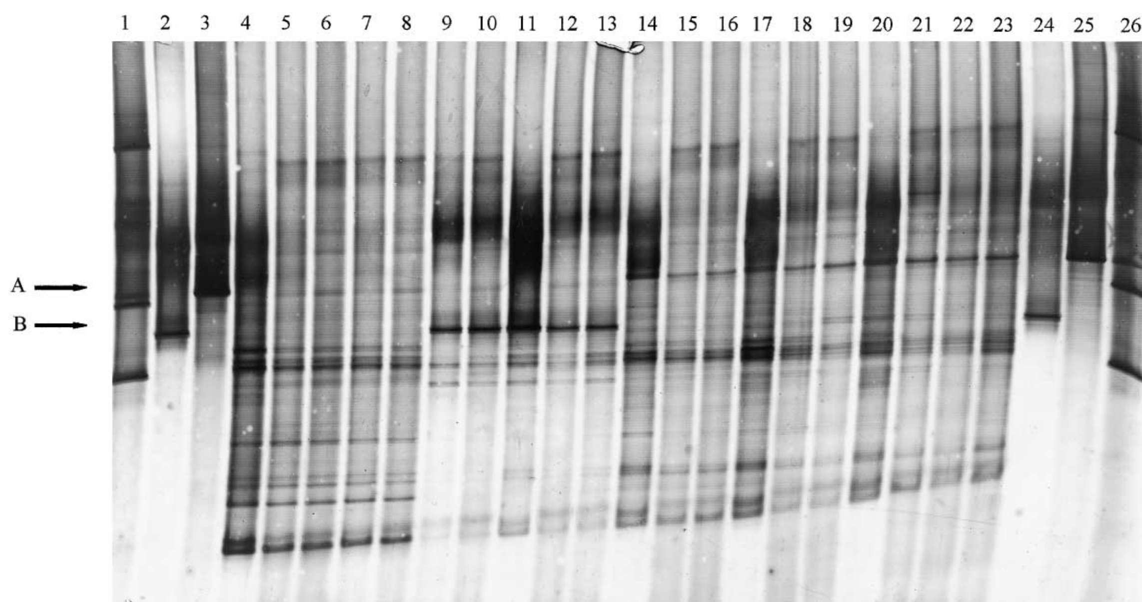


Fig. 2. DGGE analyses of bacterial community structure of tomato roots on the basis of 16S rDNA targeted PCR of DNA extracts. Lanes 1 and 26, marker (from top to bottom, amplicons of *Enterobacter cloacae* BE1, *Rhizobium leguminosarum* bv *trifolii* R62, *Arthrobacter* sp., *Burkholderia cepacia* P2); lanes 2 and 24, *R. solanacearum* 1609 pure culture (arrow marked B); lanes 3 and 25, *P. corrugata* IDV1(*chr::gfp*1) pure culture (arrow marked A); lanes 4–8, plants from untreated soil; lanes 9–13, plants from soil with *R. solanacearum* 1609; lanes 14–18, plants from soil with *P. corrugata* IDV1(*chr::gfp*1); lanes 19–23, plants from soil with *R. solanacearum* 1609 and *P. corrugata* IDV1(*chr::gfp*1).

Surprisingly, the GFP–CFU numbers of strains IDV1(*chr::gfp*1) and UA5-40G2 were similar in bulk and rhizosphere soils throughout, with only an ephemeral difference in bulk soil after 8 days, which disappeared after 14 days. The counts in the rhizoplane obtained after 8 and 14 days were, with one exception (treatments h and i, 14 d), also statistically similar between corresponding treatments with the two strains (Table 2). However, after 8 days strain IDV1 (*chr::gfp*) often showed a trend towards lower counts than strain UA5-40G2 (treatments d–g, rhizoplane), but this was reversed after 14 days, and strain IDV1(*chr::gfp*) showed apparent growth. The higher GFP–CFU numbers in rhizosphere than in bulk soil compartments demonstrated the stimulating effect of tomato roots on the survival of both strains (Table 2). These observations indicate that both strains had the capacity to survive and colonize tomato roots, but that strain IDV1 was more likely to exert its (beneficial) activity in this process than strain UA5–40G2.

IFC counts of *R. solanacearum* 1609 in inoculated bulk and rhizosphere soils and the rhizoplane (Table 2, treatments b, c and h–k) ranged from log 6.87 to 7.77 (day 8, bulk soil), log 7.21 to 7.79 (day 14, bulk soil), log 7.01 to 8.75 (day 8, rhizosphere soil), log 7.15 to 8.95 (day 14, rhizosphere soil), log 7.41 to 8.75 (day 8, rhizoplane) and log 6.59 to 8.80 (day 14, rhizoplane) CFU per gram of dry soil or per gram of fresh root, respectively. Strain 1609 IFC counts from bulk soils of these treatments were similar, irrespective of the presence of the biocontrol strains. On the other hand, the strain 1609 IFC counts from tomato rhizosphere soils were higher than those from corresponding bulk soils.

Fig. 1. Light-emitting colonies obtained by antiserum-FITC staining (*R. solanacearum* 1609; small colonies) and GFP expression from *P. corrugata* IDV1(*chr::gfp*1) [a] and *P. fluorescens* UA5-40G2 [b] (both larger colonies) obtained from tomato rhizosphere soil treated with pathogen strain 1609 and either of the two control strains.

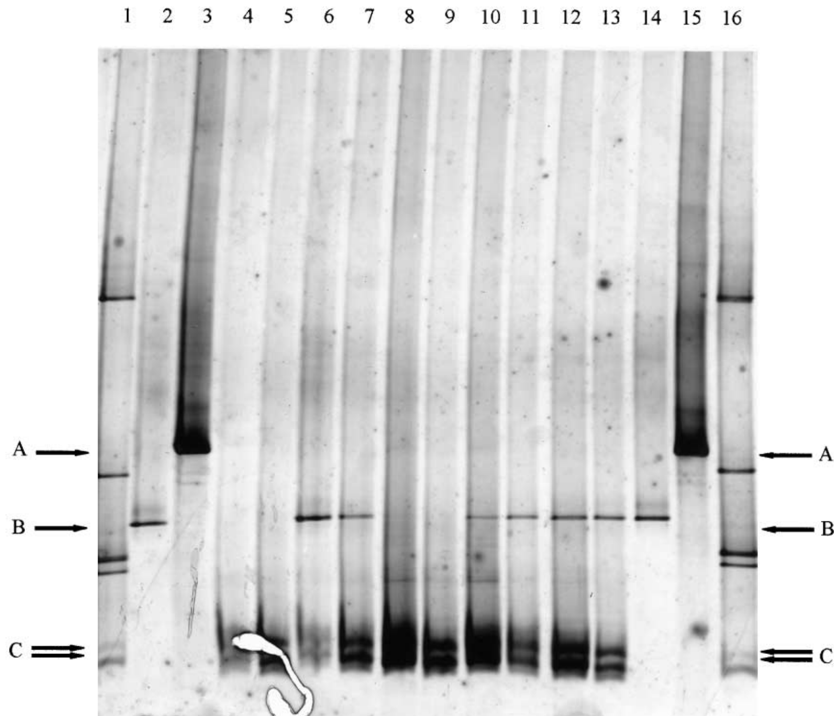


Fig. 3. DGGE analyses of bacterial community structure of tomato stems on the basis of 16S rDNA targeted PCR of DNA extracts. Lanes 1 and 16, marker (see legend of Fig. 2); lanes 2 and 14, *R. solanacearum* 1609 pure culture (arrow marked B); lanes 3 and 15, *P. corrugata* IDV1(*chr::gfp*1) pure culture (arrow marked A); lanes 4 and 5, plants from untreated soil; lanes 6 and 7, plants from soil with *R. solanacearum* 1609; lanes 8 and 9, plants from soil with *P. corrugata* IDV1(*chr::gfp*1); lanes 10–13, plants from soil with *R. solanacearum* 1609 and *P. corrugata* IDV1(*chr::gfp*1); double band arrow marked C in lanes 4–13 is originating from tomato chloroplasts.

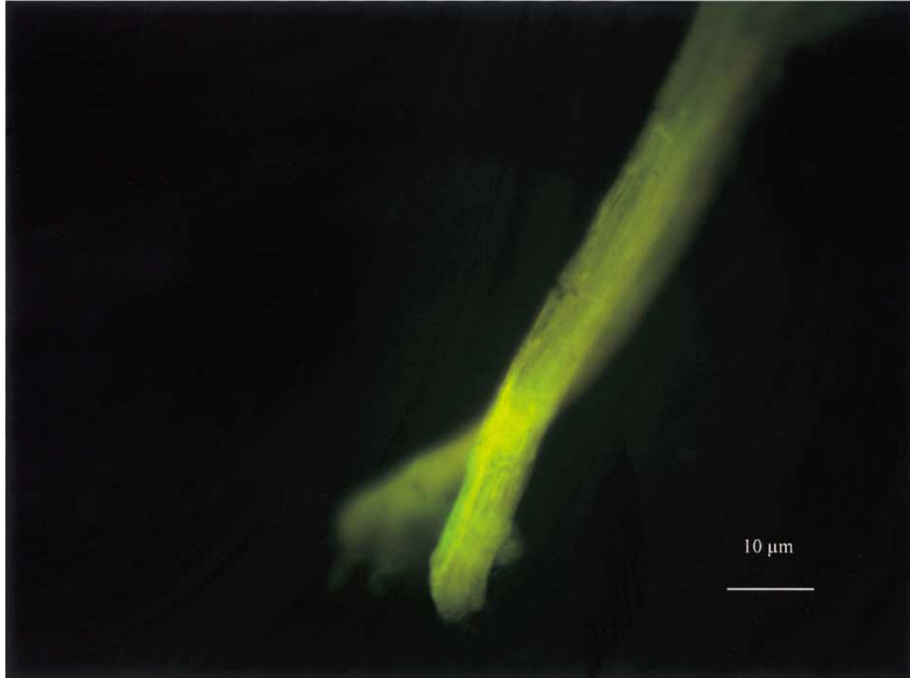
Dipping of tomato roots in suspensions of either of the two biocontrol strains resulted in consistent significant reductions of strain 1609 IFC counts in rhizosphere and rhizoplane samples (Table 2). This implies that *R. solanacearum* 1609 is impaired in its colonization of tomato roots in the presence of either biocontrol strain. Suppression of *R. solanacearum* 1609 was most pronounced as a result of the presence of strain IDV1(*chr::gfp*1), indicating the stronger antagonistic activity of this organism towards the pathogen.

3.3. *R. solanacearum* strain 1609 detection in tomato stem parts by PCR and IFC

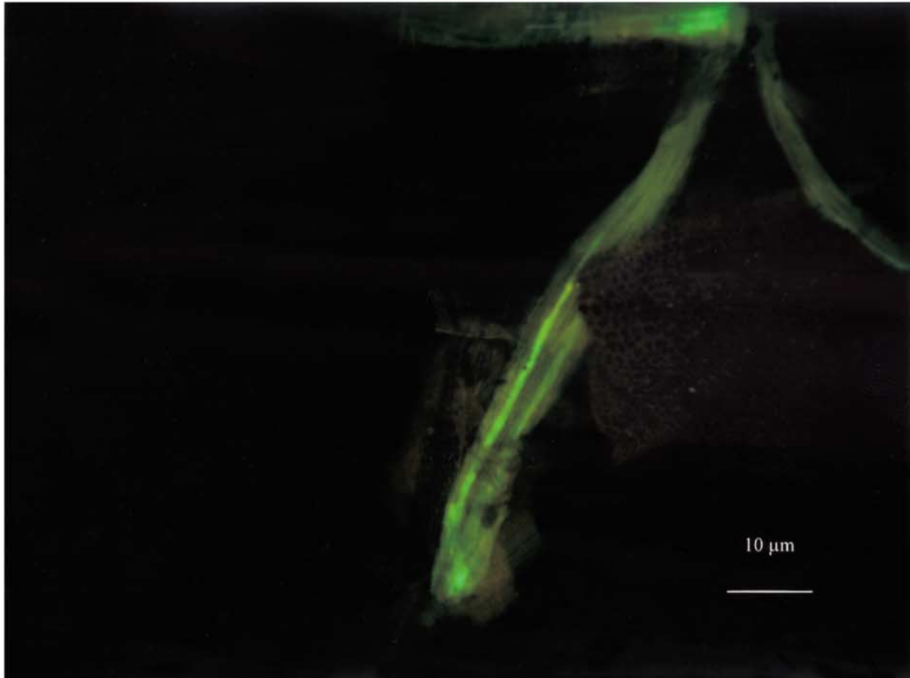
PCR amplification of DNA extracts from stems of tomato plants grown in untreated systems, with primers D2 and B, did not yield the *R. solanacearum*-specific amplicon of 650 bp, indicating the absence of the *R. solanacearum* specific target sequence. *R. solanacearum*-specific IFC counts confirmed this finding. On the other hand, PCR amplification and

Fig. 4. Fluorescent in situ hybridization of *p*-formaldehyde-treated tomato roots using probes EUB338-FITC (universal probe, green light emission) (a, b and d) and RSOLB-Cy3 (*R. solanacearum* directed probe, red light emission) (c). Tomato plant roots were either grown in untreated soil (a) or in soil with *R. solanacearum* 1609 and *P. corrugata* IDV1(*chr::gfp*1) (b–d). No signal was obtained with untreated roots hybridized with probe RSOLB (a), whereas *R. solanacearum* 1609 cells were shown to be present in the vascular cylinder (b and c) and *P. corrugata* IDV1(*chr::gfp*1) cells at the surface of tomato roots (d).

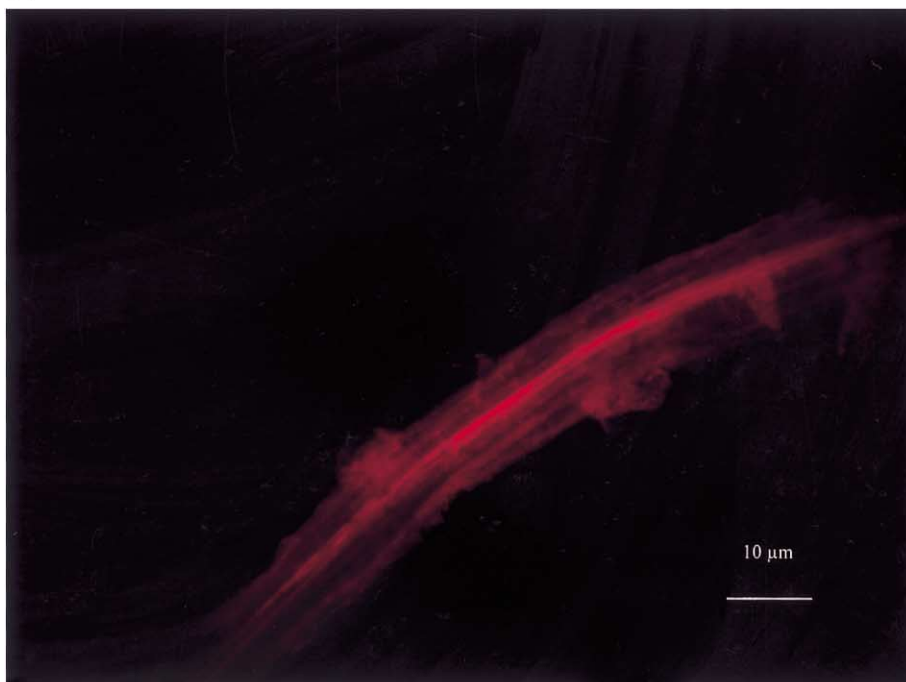
(a)



(b)



(c)



(d)

Fig. 4 (*continued*).

IFC detection were mutually supportive in the detection of *R. solanacearum* strain 1609 in infected plants (Table 1).

Using these methods, even healthy looking plants gave positive signals (Table 1, treatment b), which demonstrated the occurrence of symptomless infections. Such symptomless infections were mainly observed in plants that had grown in soil containing either one of the two biocontrol strains next to strain 1609. Specifically, in systems in which the control strains had been added to the soil but not to the roots, strain 1609 was able to invade the stem tissue of all plants, whereas only a fraction of the plants which had their roots initially dipped in cell suspensions of the biocontrol strains was invaded (Table 1). In particular in the treatment in which strain IDV1(*chr::gfp*1) had been added both to soil and roots (Table 1, treatment j), only two out of six plants showed the presence of the pathogen in their stem tissue, as evidenced by both *R. solanacearum*-specific IFC and PCR. This highlights the apparent antagonistic activity of strain IDV1(*chr::gfp*1), which is not only able to attenuate tomato root colonization by *R. solanacearum* 1609 (Table 2), but also to reduce the severity of pathogen invasion of tomato plants from soil.

3.4. Assessment of bacterial community structure and detection of R. solanacearum 1609 and P. corrugata IDV1(chr::gfp1) in the rhizoplane and stems of tomato plants by PCR–DGGE

A consistent pattern of roughly 15 strong bands at different migratory distances, was observed after PCR–DGGE analysis of root DNA extracts from untreated tomato plants (Fig. 2). With exception of the strain 1609 treatments, this banding pattern was also apparent in the other rhizoplane DNA samples analyzed, i.e. of plants treated with *P. corrugata* IDV1 (*chr::gfp*1) or with this strain in combination with *R. solanacearum* 1609. The DGGE patterns obtained from the tomato stem parts were much less complex. Up to four bands appeared, of which a strong double band originated from tomato chloroplasts (Fig. 3), and, an incidentally appearing, third (weak) band were not related to the inoculant strains.

PCR–DGGE analysis on pure culture DNA from *R. solanacearum* 1609 and *P. corrugata* IDV1(*chr::gfp*1) resulted in single and distinguishable bands (Figs. 2 and 3). These bands were absent from patterns obtained from the rhizoplane and stems of untreated plants after PCR–DGGE analysis (Figs. 2 and 3, respectively). An *R. solanacearum* 1609 related band was observed on DGGE gels containing PCR products from rhizoplane DNA extracts of plants treated with *R. solanacearum* 1609 alone, or together with *P. corrugata* IDV1 (*chr::gfp*1), although the band from the latter treatment was much weaker or virtually absent. Rhizoplane DNA extracts from all strain IDV1 (*chr::gfp*1) treated plants yielded bands comigrating with the product from this strain.

Furthermore, PCR–DGGE performed with DNA extracts from root parts, sampled at different zones along the root axis (from soils with strain IDV1 (*chr::gfp*1) and with the combination of strains 1609 and IDV1(*chr::gfp*1)) suggested the presence of strain IDV1(*chr::gfp*1) throughout (not shown), indicating that this biocontrol strain was ubiquitously present along the root.

R. solanacearum 1609 specific bands were observed by PCR–DGGE of stem DNA extracts from tomato plants treated with *R. solanacearum* 1609 alone, or in combination with strain IDV1(*chr::gfp*1) (Fig. 3). The presence of this band in all samples demonstrates the invasive properties of the pathogen, irrespective of whether biocontrol strain IDV1 (*chr::gfp*1) colonized the roots or not. PCR–DGGE on stem DNA extracts of treated plants did not reveal a band related to strain IDV1(*chr::gfp*1), indicating that this strain did not become endophytic and that its antagonistic action is likely to take place in the tomato rhizosphere or rhizoplane.

3.5. Fluorescent in situ hybridization

Roots (internal parts) of untreated tomato plants did not show a signal with either probe RSOLB (specific for *R. solanacearum* bv 2) or probe EUB338 (general probe for bacteria) (Fig. 4a) in FISH analysis, indicating the absence of *R. solanacearum*-like or other target organisms in these plant environments. However, roots from plants grown in soil with *R. solanacearum* 1609 revealed a clear emission of red (RSOLB) or green light (EUB338) from the vascular cylinder following FISH (Fig. 4b and c, respectively). This indicated the invasion of the xylem vessels of tomato plants by the pathogen from soil. FISH analysis of roots of strain

IDV1(*chr::gfp*1)-treated tomato plants using probe EUB338 revealed green fluorescence only at the root surface (Fig. 4d), whereas the control (untreated plants) showed much lower fluorescence (not shown). In this case, no signal was observed with probe RSOLB. This observation confirms that the localization of strain IDV1(*chr::gfp*1) was restricted to the surface of tomato roots.

4. Discussion

Assessment of the presence of *R. solanacearum* 1609 in plants merely by scoring of disease symptoms and CFUs would have given only a superficial picture of the actual invasive properties of this organism. This is highlighted by the detection of immunostainable colonies and *R. solanacearum*-specific amplicons in stem extracts of healthy plants (Table 1, Fig. 2). Latency of infection was, thus, shown to occur, in spite of the incubation of the plants at temperatures over 21 °C, the minimally required temperature for the observation of brownrot symptoms in tomato (Graham and Lloyd, 1978). The consequence of symptomless invasion of tomato plants by *R. solanacearum* is, without any doubt, the survival of the pathogen resulting in the potential for infestation of soil and other plants. That this behavior is not restricted to strain 1609 was demonstrated by Trigalet (Trigalet, personal communication). Strain 1609 appeared to be most virulent amongst a range of biovar 2 isolates from all over the world. The fact that even a highly virulent *R. solanacearum* isolate can become latent under certain conditions during infection, suggests that latency is a common feature of *R. solanacearum* behaviour in the interactions with its host. In the light of the latency conundrum and the potential for occurrence of VBNC cells (Van Elsas et al., 2000; 2001), sophisticated methods are required to assess pathogen fate and invasiveness.

The endophytic stage of *R. solanacearum* strain 1609, thus, demanded a special focus on the detection methods used. The presumptive evidence for the presence of strain 1609 in tomato stems obtained by PCR was consistently confirmed by IFC (Table 1), indicating the value of PCR detection for the rapid and reliable screening of plants for the presence of *R. solanacearum*. Moreover, Garbeva et al. (2001) recently demonstrated the potential of PCR–DGGE for the DNA-based detection of bacteria in planta. In the current study, PCR–DGGE was sufficiently powerful to reveal the presence and localization of *R. solanacearum* 1609, and the putative interaction between pathogen and biocontrol strains, inside plant tissue (Fig. 3). The localization of the pathogen and one control strain was demonstrated by in situ hybridization; whereas strain 1609 colonized the vascular tissue, strain IDV1(*chr::gfp*1) was confined to the root surface (Fig. 4). In studies with other biocontrol agents, these were apparently able to enter root tissue (Troxler et al., 1997). This possibly allowed competition with the pathogen in the xylem vessels, the plant tissue in which *R. solanacearum* thrives (Araud-Razou et al., 1998; Etchebar et al., 1998). Plant tissue invasion was not apparent for either of the two potential control strains used in the current study. However, to better assess such interactions, parallel simultaneous detection of *R. solanacearum* and the antagonist in microenvironments within plant tissue is required.

To simulate the infectious process and possible control measures, the pathogen was applied to soil, whereas the biocontrol strains were administered via direct soil inoculation or a combination of direct inoculation and root dipping. Colonization of the roots by these strains was determined by using growth-based and molecular detection methods. For CFU detection, interaction of the biocontrol strains with *R. solanacearum* on agar was a major concern. However, colonies of strain 1609 on the one hand, and of strains IDV1(*chr::gfp*1) or UA5-40G2 on the other hand, coexisted when embedded in agar (Fig. 1). Also, strain 1609 IFC counts from mixed inoculated samples did not show a sharp reduction in comparison with samples inoculated with only the pathogen. Molecular detection of *R. solanacearum*, by direct PCR amplification (Table 1) and by PCR–DGGE (Fig. 3), either when introduced singly or in combination with strain IDV1(*chr::gfp*1) (Fig. 2), supported the observations made by IFC.

The rhizosphere of tomato plants appeared to be a favourable niche for both the pathogen and the two potential biocontrol strains, as demonstrated by the observation of higher CFU numbers in the rhizosphere than in corresponding bulk soils (Table 2). However, the lower *R. solanacearum* strain 1609 IFC counts in

tomato rhizosphere soil containing either of both antagonists, compared to the counts in rhizospheres of untreated plants, indicated the occurrence of competition between pathogen and antagonist strains. This effect was clearest when roots were dipped in suspensions of the antagonists prior to planting. Furthermore, the fact that strain 1609 IFC counts in rhizosphere soil and the rhizoplane were more reduced in the presence of strain IDV1(*chr::gfp*1) than in that of UA5-40G2 indicated that strain IDV1 exerts stronger in situ antagonism against *R. solanacearum* 1609 than strain UA5-40. The antagonistic activity exerted by strain IDV1 was supported by PCR–DGGE analysis of the tomato rhizoplane. Systems containing strains 1609 and IDV1(*chr::gfp*1) (Fig. 2) showed much less intense bands indicative of strain 1609 than systems which only contained the pathogen. Any bias resulting from competition during PCR between target amplicons was excluded, as equal band intensities were observed on DGGE after amplification of mixed pure culture DNA samples from both strains (data not shown). The antagonistic action of strain IDV1(*chr::gfp*1) was not sufficient for a complete arrest of the activity of *R. solanacearum* near the roots, which may be due to the much lower numbers of the antagonist versus the pathogen. On the basis of these observations, we suggest that exclusion of the pathogen from the root surface—the port of entrance of *R. solanacearum* to the plant—via amendment with strains like IDV1 (*chr::gfp*1) or, to a lesser extent UA5-40G2, is a viable strategy, but that a number of unknown confounding factors affect the extent to which exclusion is achieved.

The niche occupation at the plant surface by strain IDV1(*chr::gfp*1) was different from that of strain 1609, as evidenced by FISH analysis (Fig. 4). Colonization at different locations along the root axis by strain IDV1 (*chr::gfp*1), as determined by PCR–DGGE, did not show any declining or decreasing trend, whereas *R. solanacearum* strain 1609 clearly invaded the plant vascular tissue (Fig. 4). Strain IDV1(*chr::gfp*1) was only capable of colonizing the root surface but not, to any substantial extent, the internal parts of the roots. As suggested before, the action of strain IDV1(*chr::gfp*1) towards strain 1609, thus, likely depended on antagonism at the root surface. We hypothesize that once the pathogen breaks through the 'barrier' formed by strain IDV1(*chr::gfp*1) microcolonies at the root surface, it may be capable of freely invading plant tissue. This hypothetical barrier might be called the "activity niche" of strain IDV1. The absence of an IDV1(*chr::gfp*1) specific band, but the presence of one from strain 1609 in DGGE gels prepared from tomato stem DNA (Fig. 3) also indicated the limitation of the "activity niche" of the antagonist.

Latent presence of strain 1609 in tomato plants was found following treatments of plants with strain 1609 alone, or in combination with strains UA5-40G2 and IDV1(*chr::gfp*1) (Table 1). An explanation may be that both biocontrol strains affect the expression of virulence genes in *R. solanacearum* cells. Induction of *R. solanacearum* virulence genes requires the accumulation of 3-hydroxypalmitic acid methyl ester (Clough et al., 1994, 1997). Any compound released by strains UA5-40G2 or IDV1(*chr::gfp*1) that would interfere with this regulatory system might delay the onset of the expression of genes involved in pathogenicity. Latent presence in plants which were treated with strain 1609 alone might even be induced by the indigenous microflora in the tomato rhizoplane. The potential role of these bacterial species in the suppression of disease by *R. solanacearum* has been established by Shiomi et al. (1999).

The polyphasic approach for detection of the pathogen and the biocontrol strains allows the precise description of the potential interactions of these organisms in association with the host plants. Improvements of the methods employed may even enhance the specificity of detection, such as indicated by the application of bacterial subgroup-specific primers for PCR–DGGE analysis (Schönfeld, unpublished results). This allows to screen for interaction between the pathogen and its phylogenetically nearest relatives. A selection of potential antagonists either obtained from the pathogen by mutation of virulence genes (Etchebar et al., 1998) or directly from the microflora of plant roots (Shiomi et al., 1999) can thus be tested under relevant conditions prior to application to open fields.

Acknowledgements

We thank Robert Zablotowicz, Sören Molin and Janet Jansson for supplying strains UA5-40, pJB29-*gfp*-mut3b and pUT*gfp*, respectively, Ineke de Vries and Rogier Doornbos for technical assistance and

Andre Trigalet, Jens Schönfeld and Joeke Postma for helpful discussions. This work was supported by the EU-FAIR program (acronym FATE, PL97-3632) and a NATO collaborative research grant to J.D.v.E., J.T.T. and J.K.

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