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Immunofluorescence colony-staining (IFC) for detection and quantification of *Ralstonia (Pseudomonas) solanacearum* biovar 2 (race 3) in soil and verification of positive results by PCR and dilution plating

J.M. van der Wolf, S.G.C. Vriend, P. Kastelein, E.H. Nijhuis, P.J. van Bekkum and J.W.L. van Vuurde
DLO Research Institute for Plant Protection (IPO-DLO), P.O. Box 9060, 6700 GW, Wageningen, The Netherlands (Phone: 31 317 476024; Fax: 31 317 410113; E-mail: j.m.vanderwolf@plant.wag-ur.nl)

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Abstract

A procedure was developed for specific and sensitive quantitative detection of *Ralstonia (Pseudomonas) solanacearum* biovar 2 (race 3) in soil. It is based on immunofluorescence colony-staining (IFC) followed by confirmation of the identity of fluorescent colonies by PCR-amplification or dilution plating on a semi-selective medium, SMSA. Addition of sucrose and the antibiotics cycloheximide and crystal violet to the non-selective tryptcase soy broth agar resulted in increased colony size and staining intensity of *R. solanacearum* in IFC. Verification of IFC-results by picking cells from IFC-positive colonies followed by dilution plating of the suspended cells on SMSA was highly efficient. The success rate was 92% and 96% with 'spiked' and naturally contaminated soils respectively. Several other bacterial species which cross-reacted with polyclonal antibodies in IFC also grew on SMSA and were difficult to distinguish from *R. solanacearum*, thereby necessitating confirmation of the results. Rapid verification of IFC-positive results directly by PCR-amplification with primers D2/B specific to division 2 of *R. solanacearum* had a success rate of 86% and 96% with 'spiked' and naturally contaminated soil samples, respectively. Primers D2/B reacted with all *R. solanacearum* division 2 strains, and strains of *R. syzygii* and the banana blood disease bacterium, but not with saprophytic bacteria cross-reacting in IFC with *R. solanacearum* antibodies. In comparative tests, IFC was able to detect consistently ca. 100 cfu g^{-1} of soil, a detection level similar to that found with direct plating on SMSA, but less laboriously, whereas detection level with a bioassay on tomato plants was only 10^4–10^5 cfu g^{-1} of soil.

Introduction

Ralstonia (Pseudomonas) solanacearum biovar 2, race 3 is the causal organism of brown rot in potato, a disease present in many potato producing areas world wide. In the last decade, brown rot outbreaks occurred in several countries in Europe, resulting in the introduction of stringent quarantine measures coupled with intensive surveys. Under cool European conditions, infected seed tubers were shown to play a major role in survival and long distance transmission of the disease (Elphinstone, 1996). Dissemination of the pathogen through contaminated surface water is an important pathway of disease transmission as infection can readily occur when contaminated water is used for irrigation (Olsson, 1976; Stead, 1996).

Infected aquatic roots of bittersweet, *Solanum dulcamara*, growing along waterways, play a role in overwintering of the pathogen and as an inoculum source, when bacteria leach out from the root system (Olsson, 1976). In Western Europe, infection of potato ground keepers and non-aquatic weeds has rarely been found and play no significant role in the epidemiology of the pathogen (Elphinstone, 1996).

Little is known about survival of *R. solanacearum* in soil under cool European conditions. Under temperate conditions in Australia, the brown rot pathogen was detected in soil for not more than two years (Shamsuddin et al., 1979; Graham and Loyd, 1979). However, it is often difficult to assess whether populations have been really extinguished. In general, detection of bacteria in soil is complicated by the low numbers of target bacteria, the high microbial background and an irregular distribution in the field.

For detection of *R. solanacearum* in soil, sensitive indicator plants such as potato, have been used by planting in test soil (Graham and Lloyd, 1978). In this way, a relatively large soil volume can be tested and only viable virulent cells are detected. A bioassay, however, is time-consuming, lacks the possibility for quantification and sensitivity was found to be ca. 10^4 cfu g^{-1} (Graham and Lloyd, 1979). For quantitative estimations, dilution plating on a semi-selective medium was used, but the high populations of non-target bacteria present in soil easily tend to overgrow the target bacteria, resulting in a detection level of 10^4 cfu g^{-1} of soil (Graham and Lloyd, 1979). Moreover, recognition of the target colonies on plates can be complicated by the presence of bacteria with a colony appearance similar to that of *R. solanacearum*. Recently, a more selective medium, SMSA (Elphinstone, 1996), has been developed which improved detection of *R. solanacearum* in soil by dilution plating but still suffered from the same drawbacks as former growth media (unpublished results).

Immunofluorescence colony-staining (IFC), which combines bacterial colony-growth and serological methods, allows sensitive and quantitative detection of several plant pathogenic bacteria in complex environments with high microbial backgrounds, such as potato peel extracts and cattle manure slurry (Van Vuurde, 1987; Van Vuurde and Roozen, 1990; Jones et al., 1994; Van der Wolf et al., 1996). Both monoclonal and polyclonal antibodies produced against *R. solanacearum* show cross-reactions with taxonomically related bacteria such as the causal organism of the banana blood disease and *R. syzygii*, but also with more distantly related soil saprophytes (Griep et al., 1998). The use of serological methods for the quarantine pathogen *R. solanacearum* should therefore be considered with care. This is particularly true for detection in soil, where populations of *R. solanacearum* may be low and high numbers of saprophytic soil bacteria are present, some of which could cross-react with the antibodies.

This paper aims at the development and evaluation of a reliable IFC-procedure for quantitative detection of *R. solanacearum* in soil in conjunction with verification procedures for IFC-positive results. Two verification strategies have been investigated: (1) confirmation of the identity of IFC-positive colonies by reisolation on the semi-selective medium SMSA followed by characterisation of *R. solanacearum*-type colonies by immunofluorescence cell-staining, (2) direct confirmation of fluorescent colonies by PCR-amplification, using primers against 16S rDNA sequences specific to division 2 of *R. solanacearum* (Boudazin et al., 1999).

Materials and methods

Bacterial strains and growth conditions. Bacterial strains, including 50 *R. solanacearum* strains (Figure 1) and 32 cross-reacting bacteria selected from previous studies (Table 2), were preserved at −80 °C on beads in 15% glycerine, 8 mg ml^{-1} Lab Lemco broth (Oxoid) (Protect, Biotrading, Wilnis, NL). *R. solanacearum* was grown on Trypticase Soy Agar (TSA, BBL) for 48–72 h at 27 °C and subsequently suspended in sterile demineralised water, in which it was stored for a maximum of 1 month at 4 °C. For other bacteria, master plates were prepared by growing bacteria from the beads on TSA for 48–72 h at 27 °C. Master plates were stored for up to 1 week at 17 °C. Prior to use, bacteria were grown from water or master plates on slopes of Growth Factor Agar (GF; 2.3 mM K_2HPO_4, 0.2 mM $MgSO_4 \cdot 7H_2O$, 1.7 mM NaCl, 4.3 mM $NH_4H_2PO_4$, 5 mM glucose, 0.5% yeast (Difco) and 1.5% agar, pH 7.2) for 24 h at 27 °C. IPO strain no. 1609 (Dutch Plant Protection Service (PD) strain no. 2763) was used as a reference strain. Bacterial suspensions were prepared in a quarter strength Ringer solution.

Dilution plating on SMSA. The semi-selective SMSA-medium as described by Engelbrecht (1994) and modified by Elphinstone et al. (1996) was used. A volume of 50 µl of test material was plated with using a spiral plater (DW Scientific, IKS, Leerdam, The Netherlands), creating a 20-fold gradient per plate.

Tomato bioassay. The bioassay in tomato plants (cv. Moneymaker) was done as described by Janse (1988).

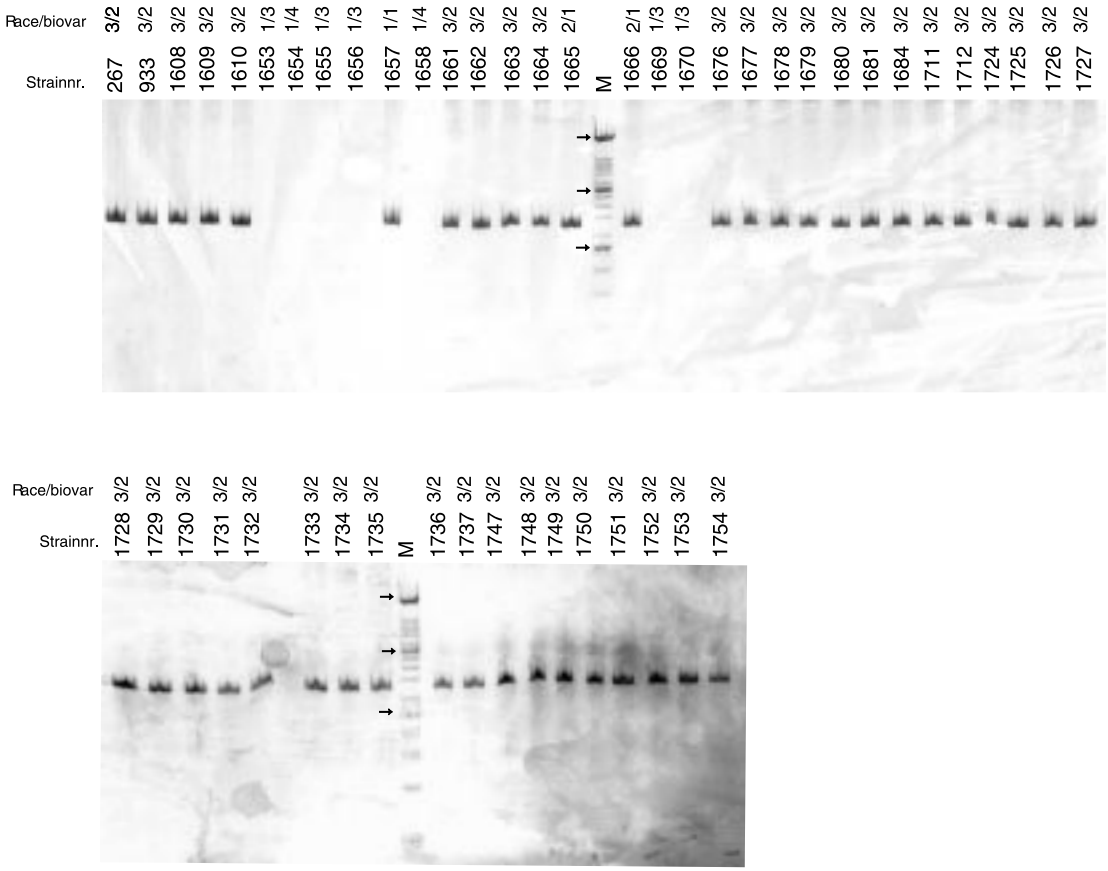

Figure 1. Reaction of 39 race 3, biovar 2 strains; 1 race 1, biovar 1 strain; 3 race 1, biovar 3 strains; 2 race 1, biovar 4 strains and 2 race 2, biovar 1 strains of *R. solanacearum* in PCR-amplification using primers against 16S rDNA sequences specific for division 2. The arrows indicate the 500, 1000 and 1500 bp bands of the 100-bp marker (M).

Young tomato plants at the 3–4 leaf stage were stem inoculated with sample extracts and incubated in a glass house for 16 h at 28 °C during day time and for 8 h at 24 °C at night at a relative humidity of 70–80%. Plants were observed weekly for wilting symptoms over a period of five weeks.

Preparation of soil extracts. A *R. solanacearum*-free clay soil and a loamy sand soil as determined by IFC were used for model studies with added bacteria. Soils were stored at 6 °C, and one day prior to the experiment water was added to 85% moisture level and allowed to stand for 1 day at room temperature for the soil microflora to stabilise itself. Naturally contaminated clay soil, loamy sand soil and organic sand soil were collected from different parts of the Netherlands and tested directly on arrival. Ten g of soil was added to 100 ml of 0.85% NaCl solution and 10 g of coarse gravel with an average diameter of 3 mm and shaken for 20 min (280 rpm) at 27 °C. Extracts were filtered through cotton wool. In the case of seeded samples, *R. solanacearum* strain 1609 was grown for 24 h at 27 °C, suspended and diluted in sterile water, and added to the soil just before extraction.

Antibody production. Antiserum 9527BCD was produced against five race 3 strains (IPO strain nos. 267, 268, 1608, 1609 and 1610), inoculated in equal numbers in the rabbit. Antibodies were purified as described by Steinbuch and Audran (1969) and conjugated with fluorescein isothiocyanate (FITC) as described by Allen and Kelman (1977).

Immunofluorescence cell-staining. Direct immunofluorescence cell-staining was carried out on *R. solanacearum*-type colonies on SMSA-plates. Bacteria were diluted in sterile demineralised water and tested in concentrations of ca. 10^7 cells ml^{-1} as described by Van der Wolf et al. (1993) using antiserum 9527BCD conjugated with FITC in a 100-fold dilution.

Immunofluorescence colony-staining. IFC was performed as described by Van Vuurde and Roozen (1990) with some modifications. In a 16 mm well of a 24 well tissue culture plate, 100 µl of sample extract (undiluted and 1 : 10 in a quarter strength Ringer solution) was mixed with 300 µl of molten agar medium at 48 °C using an ELISA plate shaker. Plates were incubated for 2–3 days at 27 °C and successively washed for 3 h with 1 ml of sterile demineralised water to remove surface grown colonies. Colonies were stained with 300 µl FITC-conjugated antiserum 9527BCD, 50-times diluted in PBS (0.27 M NaCl, 0.04 M Na_2HPO_4, 2.9 mM KH_2PO_4, 5.4 mM KCl) supplemented with 0.1% Tween 20. IFC-preparations were washed for 24 h with 300 µl washing buffer (10-times diluted PBS supplemented with 0.1% Tween 20) refreshing the buffer after 1, 2 and 6 h. Fluorescent colonies were observed with an epi-fluorescent stereo microscope equipped with a GFP plus filter pack and epifluorescent illumination with high pressure lamp (Leica).

The basal agar medium used in IFC consisted of 10% TSBA (3 g l^{-1} of trypticase soy broth (BBL) and 10 g l^{-1} of agar (Oxoid)) supplemented with 0.1 mg ml^{-1} cycloheximide to suppress fungal growth. To improve the staining quality and growth of *R. solanacearum* several combination of additives as described in Table 1 were tested: sucrose, a sugar which can be utilised by *R. solanacearum* but not by a taxonomically related species such as *R. pickettii* (Anonymous, 1984), and three antibiotics: rifampicin for specific suppression of *Bacillus cereus var. mycoides*, a fast growing fungus-like bacterial species, crystal violet to suppress Gram-positive bacteria and chloramphenicol which inhibits protein synthesis.

Bacteria from fluorescent and non-fluorescent colonies were collected with a capillary needle and suspended in 300 µl of demineralised water before plating on SMSA and PCR testing. In order to reduce the risk of contamination with non-target bacteria, only fluorescent colonies were picked that were distant from non-fluorescent colonies.

Polymerase chain reaction. Suspensions of pure isolates were tested at a concentration of 10^6 cells ml^{-1} in 5 mM NaOH. IFC-samples were diluted 1 : 1 with 10 mM NaOH. Samples were boiled for 5 min and centrifuged for 3 min at 12 000*g* prior to PCR. Ten µl of the supernatant was tested in a total volume of 50 µl PCR reaction mix. The PCR-reaction mix contained 0.2 µl of Taq polymerase (1 unit; Boehringer), 5 µl of 10× PCR-buffer (Boehringer), consisting of 10 mM Tris–HCl (pH 8.3), 50 mM KCl, 1.5 mM $MgCl_2$, 0.1 mg ml^{-1} gelatine, 5 µl (0.2 mM) dNTPs (Pharmacia), 0.5 µl (400 ng) of each primer, 29.6 µl of demineralised water. *R. solanacearum* division 2 specific forward primer D2 (5′-GTCCGGAAAGAAATCGCTTC-3′) (position 405–424) and the non-specific reverse primer B (5′-GCACACTTCATCCAGCGA-3′) (position 1064–1046) were derived from 16S rDNA sequences (Boudazin et al., 1999; Seal et al., 1999). A total of 35 PCR cycles were run on a Perkin Elmer 9600 thermocycler under the following conditions: denaturation for 20 s at 94 °C, primer annealing for 20 s at 66 °C and DNA extension for 30 s at 72 °C.

Amplicons were analysed on a 1% agarose gel and stained with ethidium bromide (Sambrook et al., 1990). Alternatively, amplicons were separated on a 5% horizontal polyacrylamide gel using the Multiphor II system (Pharmacia) and stained with the BioRad silver staining procedure according to the manufacturer's instructions.

Statistical analysis. The effect of additives in TSBA on percentage recovery of soil bacteria and *R. solanacearum* was analysed by computing the least significant difference (LSD) using the statistical package GENSTAT 5.3.

Results

Improvement of agar media for use in IFC. IFC-detection of *R. solanacearum* race 3 strains 267, 1608 and 1609 added to undiluted clay and loamy sand soil extracts using 10% TSBA supplemented with cycloheximide to suppress fungal growth, resulted in a percentage recovery between 51% and 97% (Table 1). Colonies were small and weakly stained, particularly in loamy sand soil.

To improve the staining quality and growth of *R. solanacearum*, the effect of several combinations of additives were tested (Table 1). A significant improvement in percentage recovery, colony size and staining

Table 1. Effect of selective compounds added to 10% TSBA on growth and colony fluorescence of *Ralstonia solanacearum* strains 267, 1608 and 1609 in extracts of clay soil and loamy sand, pour plated for IFC, and on percentage recovery of soil bacteria

Additions to 10% TSBA	Percentage recovery of soil bacteria[a]		Percentage recovery of *R. solanacearum*[b]						Average colony size (μm)		Colony fluorescence[c]	
			Clay soil			Sandy loam						
	Clay soil	Sandy loam	IPO267	IPO1608	IPO1609	IPO267	IPO1608	IPO1609	Clay soil	Sandy loam	Clay soil	Sandy loam
Cy[d]	ND	ND	85.0[b]	78.8	97.1	51.2	52.5	50.6	36	4	3	2
Cy + Rp	44.3	41.5	8.0	50.8	53.3	ND	28.4	20.6	46	4	2	2
Cy + Cm	58.8	54.9	50.6	53.0	55.1	15.1	35.0	36.8	39	4	4	2
Cy + Cv	2.8	37.6	56.0	57.5	61.3	24.2	26.3	30.6	46	4	4	2
Cy + Cm + Cv	2.7	29.0	49.8	55.1	57.5	11.4	28.9	31.8	43	4	3	2
Cy + Cm + Su	61.6	56.8	57.6	59.5	64.1	9.5	42.8	46.3	43	4	4	2
Cy + Cv + Su	3.0	30.3	74.6	84.1	83.6	70.5	75.6	80.8	50	25	4	4
Cy + Cm + Cv + Su	2.8	30.3	54.7	59.1	59.7	16.5	38.6	38.5	46	4	3	2

[a]Number of cfu of bacteria in 10% TSBA without additives was considered as 100% (both in clay soil and loamy sand 2×10^6 cfu g^{-1} of soil were found). LSD = 10.20 (interaction soil × medium), $n = 3$.

[b]Number of cfu of pure cultures of *R. solanacearum* in 10% TSBA without additives was considered as 100%. LSD = 9.3 (interaction strain × soil × medium), $n = 3$.

[c]2 = weak fluorescence, 3 = moderate fluorescence, 4 = strong fluorescence.

[d]Cy = Cycloheximide 100 mg l^{-1}; Rp = Rifampicin 0.5 mg l^{-1}; Cm = Chloramphenicol 5 mg l^{-1}; Cv = Crystal violet 5 mg l^{-1}; Su = Sucrose 1 g l^{-1}.

quality in loamy sand soil sample was obtained only by addition of a combination of 0.1 mg ml^{-1} cycloheximide, 5 μg ml^{-1} crystal violet and 1 mg ml^{-1} sucrose to 10% TSBA (TSBA-CCS). Although TSBA-CCS improved colony size and staining intensity for both soil types, percentage recovery was only greater with loamy sand soil but not significantly different with clay soil compared with 10% TSBA with cycloheximide (Table 1). Sucrose had a stimulatory effect on the growth of *R. solanacearum*. The addition of chloramphenicol, but not crystal violet, neutralised this effect. Crystal violet, particularly in clay soil, strongly suppressed growth of fast growing Gram-positive bacteria. However, addition of crystal violet had no significant effect on growth and staining quality of *R. solanacearum* in IFC.

Cross-reacting bacteria in IFC. A panel of 32 bacteria, selected for having shown cross-reactions in previous studies with polyclonal antisera against race 3 of *R. solanacearum* in IF and/or ELISA (Griep et al., 1998) together with the homologous target strain and *Erwinia chrysanthemi* as a negative control, were tested in IFC (Table 2). Of 32 strains, 16 were taxonomically related to *R. solanacearum*, belonging to the genera *Pseudomonas*, *Burkholderia* and *Ralstonia* and 16 more distantly related or unidentified bacteria. Twenty-two of these strains reacted in IFC, in which all but two were easily recognised on the basis of the staining intensity or an incomplete staining restricted to the edge of the colonies only (results not shown).

Verification of IFC-positive colonies. Two strategies for verification of IFC-positive colonies were evaluated with respect to specificity and efficiency: (1) isolation of bacteria on SMSA followed by confirmation by IF cell staining of colonies with a colony appearance typical of *R. solanacearum* and (2) direct PCR-amplification of bacteria from fluorescent colonies.

From the 22 strains reacting in IFC, 12 were able to grow on SMSA (Table 2). From the total panel of 32 cross-reacting bacteria, 22 isolates were able to grow on SMSA. Two *Burkholderia cepacia* strains (strain nos. 1702 and 1703), had a colony appearance similar to that of *R. solanacearum*.

Two division 2 strains of the banana blood disease bacterium and *R. syzygii* were PCR-amplified but none of the 39 saprophytic bacteria tested (Table 2). Out of 41 *R. solanacearum* division 2 strains, including 40 potato biovar 2 and one biovar 1 strain, all were PCR-positive, but none of the seven division 1 strains tested (Figure 1).

From 70 fluorescent colonies taken from IFC-preparations with clay and loamy sand soil extracts seeded with *R. solanacearum* strain 1609, 68 could be isolated on SMSA (Table 3). IF testing of the colonies on SMSA confirmed the identity of the colonies. When non-fluorescent colonies, sampled from IFC-preparations with soil extracts without added bacteria were grown on SMSA, only 4 out of 120 exhibited a colony-appearance similar to that of *R. solanacearum* (results not shown). None of these were IF-positive. In PCR, 56 out of 70 fluorescent colonies sampled were positive, all non-fluorescent colonies being negative. Colonies morphologically similar to *R. solanacearum* on SMSA and positive in IF were grown from all PCR-positive samples.

From naturally infested soils, when the identity of 24 fluorescent colonies were checked, 22 formed typical colonies on SMSA and were IF positive and 23 were PCR positive (Table 4). Cells from 10 atypically stained colonies, 7 from contaminated and 3 from non-contaminated soils, were negative in PCR and showed no growth on SMSA.

Detection levels of IFC, SMSA-dilution plating and bioassay on tomato. IFC was compared with dilution plating on SMSA and the tomato bioassay for the detection of *R. solanacearum* in soil. Serial dilutions of cultures of strains 1608 and 1609 were added to extracts of a loamy sand and clay soil samples. The detection level of IFC was found to be ca. 10 cfu ml^{-1} of soil extract, which is equivalent to 100 cfu g^{-1} of soil (Table 5). Reliable quantification was possible up to densities of 10^4 cfu per IFC-preparation. At densities above 10^6 cfu per preparation, a weak staining of target colonies was observed, probably due to competition for antibodies. The detection limit by dilution plating on SMSA was found to be 10–100 cfu ml^{-1} of soil extract. In clay soil extract without added bacteria, saprophytic soil bacteria were frequently found on SMSA plates with a colony size, colour and morphology similar to *R. solanacearum*. The detection limit for plating on SMSA was based on testing three colonies with a typical morphology and identity in IF with polyclonal antiserum 9523. In addition, a 100-fold serial dilution of the total bacterial population harvested from plates in 1 ml of demineralised water was tested in IF. *R. solanacearum* was present when at least one of the IF-tests was positive. Frequently the number

Table 2. Growth of cross-reacting bacteria[1] on SMSA and reactions of cross-reacting bacteria in IFC, and PCR-amplification based on primers D2/B against 16S rDNA sequences

IPO strain no.	Bacteria[2]	Source	Host/ substrate	Country	IFC reaction[3]	Growth on SMSA	PCR reaction
S302	*Ralstonia syzygii*	PD2093	Syzygium	Indonesia	++	−	+
S303	Banana blood disease	PD2100	Musa	Indonesia	+++	−	+
S304	Banana blood disease	PD2103	Musa	Indonesia	++	−	+
S306	*Ochrobactrum anthropi*	PD2808		The Netherlands	++	+	−
S307	*Ochrobactrum anthropi*	PD2812		The Netherlands	−	+	−
S339	*Enterobacter* sp.	IPO-DLO	Potato	The Netherlands	++	+	−
S342	*Enterobacter* sp.	IPO-DLO	Soil	The Netherlands	+	+	−
S345	*Enterobacter* sp.	IPO-DLO	Potato	The Netherlands	−	+	−
S346	Coryneform	IPO-DLO	Potato	The Netherlands	−	−	−
S347	*Rhodococcus* sp.	IPO-DLO	Potato	The Netherlands	+	−	−
S348	Unidentified	IPO-DLO	Potato	The Netherlands	−	+	−
1652	*Ralstonia* sp.	NIZO96	Milk	The Netherlands	++	+	−
1659	*Ralstonia pickettii*	PD1513	Blood	The Netherlands	++	+	−
1690	*Rathayibacter tritici*	IVIA 1580.3		Spain	−	−	−
1692	*Aureobacterium liquefaciens*	IVIA 1580.7		Spain	+	−	−
1693	*Aureobacterium liquefaciens*	IVIA 1580.10		Spain	+	−	−
1694	*Serratia fonticola*	IVIA 1534.c		Spain	+	+	−
1701	*Burkholderia caryophylli*	NCPPB353		United Kingdom	++	+	−
1702	*Burkholderia cepacia*	NCPPB353		United Kingdom	++(+)	+[4]	−
1703	*Burkholderia cepacia*	NCPPB353		United Kingdom	−	+[4]	−
1704	*Burkholderia andropogonis*	NCPPB353		United Kingdom	++(+)	−	−
1705	*Burkholderia caryophylli*	NCPPB353		United Kingdom	++	+	−
1707	*Burkholderia plantarii*	NCPPB353		United Kingdom	−	+	−
1708	*Burkholderia glumae*	NCPPB353		United Kingdom	+/−	−	−
1710	*Erwinia carotovora* subsp. *carotovora*	INRA2.33		France	+	−	−
1713	Unidentified	INRA1531		France	+/−	−	−
1714	*Pseudomonas marginalis*	CFBP1538		France	−	+	−
1715	Unidentified	INRAR3		France	−	−	−
1718	*Burkholderia cepacia*	R5d-1			+	+	−
1720	*Ralstonia pickettii*	Prr150		United Kingdom	−	−	−
1722	*Ochrobactrum anthropi*	T3b7		United Kingdom	+	+	−
1723	*Ralstonia* sp.	PD2778		The Netherlands	+	+	−
502	*Erwinia chrysanthemi*	PD266	Potato	The Netherlands	−	−	−
1609	*Ralstonia solanacearum*	PD2763	Potato	The Netherlands	++	+	+

[1]Bacteria were selected for cross-reactions with polyclonal antisera against *R. solanacearum* in IF and/or ELISA (Griep et al., 1998).
[2]Fatty acid analysis was used for strain identification and the best matches with the database of the Microbial Identification System are given. Strains were obtained from J.D. Janse (Plant Protection Service (PD), Wageningen, the Netherlands), M.M. Lopez (IVIA, Valencia, Spain), A.C. Le Roux (INRA, Rennes, France) and J.G. Elphinstone (CSL, York, United Kingdom).
[3]IFC was done with polyclonal antiserum 9523 against *R. solanacearum*. IFC signals: +++ = strong fluorescence intensity, ++ = moderate fluorescence intensity, + = weak fluorescence intensity, − = signal below background.
[4]Colony-appearance similar to *R. solanacearum*.

of colonies on SMSA-plates could not be determined due to excessive slime production.

Wilting of tomato plants could only be induced consistently at concentration of 10^4 cfu ml^{-1} and higher. *R. solanacearum* could be easily isolated from wilted plants; plating of extracts from wilted plants resulted in almost pure cultures on SMSA.

Discussion

IFC was found to be a sensitive method for quantitative detection of *R. solanacearum* (biovar 2) in soil, and IFC will be of particular value in epidemiological and ecological studies on survival of this pathogen in complex matrices in general. Additions to

Table 3. Characterisation of bacteria, picked from colonies in IFC-preparations with or without added *R. solanacearum* in soil extracts, using PCR-amplification and plating on SMSA followed by IF cell-staining of suspected colonies

Sample[a]	Total no. of sampled colonies	No. of colonies resulting in growth of (IF-positive) *R. solanacearum* typical colonies on SMSA	No. of colonies PCR-positive[c]
Fluorescent colonies from seeded clay soil	50	48[b]	43
Non-fluorescent colonies from non-seeded clay soil	50	0	0
Fluorescent colonies from seeded loamy sand	20	20	13
Non-fluorescent colonies from non-seeded loamy sand	70	0	0

[a]IFC-preparations without added target bacteria contained up to 1000 colonies. In seeded soil extracts, target bacteria were added in a ratio of 1 : 1 to 10 : 1 with soil bacteria. In IFC-preparations with plain soil extracts no cross-reacting bacteria were observed.
[b]Characterised on the basis of colony appearance and IF cell-staining.
[c]PCR-amplification based on 16S rDNA primers D2/B.

Table 4. Characterisation of bacteria, collected from fluorescent colonies in IFC-preparations with naturally infested and non-infested soil extracts[a], by SMSA plating and PCR-amplification

Appearance in IFC-preparations	Total no. of IFC-positive colonies tested	No. of colonies positive on SMSA	No. of colonies positive in PCR
Extracts from infested fields			
Typically stained[b]	24	22	23
Atypically stained[c]	7	0	0
Extracts from non-infested fields			
Atypically stained	3	0	0

[a]Soil samples were collected from three parts of the Netherlands and comprised one clay soil, a loamy sand and an organic sand.
[b]Typically stained: stained colonies similar to *R. solanacearum* in spiked samples.
[c]Atypically stained: stained colonies obviously different from *R. solanacearum* in spiked samples.

the TSBA (TSBA-CCS) resulted in an increased colony growth and staining intensity of *R. solanacearum* in TSBA-CCS compared to TSBA. The improved results with TSBA-CCS can be explained tentatively by the stimulatory effect of sucrose on the growth of *R. solanacearum*. The detection level was determined at ca. $10\,\mathrm{cfu\,ml^{-1}}$ of soil extract, equivalent to $100\,\mathrm{cfu\,g^{-1}}$ of soil, which is approximately the theoretical detection level for IFC in which 0.1 ml of soil extract is sampled. A similar detection level for IFC was found for *Erwinia carotovora* subsp. *atroseptica* in cattle manure slurry and potato tuber extract (Van Vuurde and Roozen, 1990; Jones et al., 1994). In contrast, Elphinstone et al. (1996) found consistent positive results with IFC for the detection of *R. solanacearum* in potato tuber extracts, only when 2×10^4 to 2.5×10^6 cells $\mathrm{ml^{-1}}$ were present. This was attributed to high background fluorescence and tuber tissue debris masking fluorescent colonies. This difference in detection limits might be explained by the presence of several antibiotics in the IFC agar medium used by Elphinstone et al. (1996), which, according to our results, would suppress growth in pour plates and hence drastically decreased recovery of target colonies. It is also possible that the difference in test substrates (potato tissue and soil extract) could have affected the results.

Table 5. Detection of *R. solanacearum* in loamy sand and clay soil by IFC, SMSA-dilution plating and a tomato bioassay

Density of *R. solanacearum* in cfu ml^{-1} of soil extract[a]	IFC[b]	SMSA-plating	Tomato bioassay	Density of *R. solanacearum* in cfu ml^{-1} of soil extract	IFC	SMSA-plating	Tomato bioassay
Loamy sand, IPO1608				Clay soil, IPO1608			
$>10^4$	+[d]	+	+	$>10^4$	+	+	+
$>10^4$	+	+	+	$>10^4$	+	+	+
3923 (1904)	+	+	+	6360 (517)	+	+	+
1018 (541)	+	+	+/−	902 (337)	+	+	+/−
181 (73)	+	+	−	110 (31)	+	+	−
32 (6)	+	+	−	21 (8)	+	+	−
13 (13)	+	+	−	1 (2)	+/−	+	−
1 (2)	+/−	−	−	0	−	−	−
0	−	−	−	0	−	−	−
0	−	−	−	0	−	−	−
Loamy sand, IPO1609				Clay soil, IPO1609			
$>10^4$	+[c]	+	+	$>10^4$	+	+	+
$>10^4$	+	+	+	$>10^4$	+	+	+
$>10^4$	+	+	+	$>10^4$	+	+	+
2200 (246)	+	+	+	2643 (175)	+	+	+
404 (53)	+	+	−	374 (149)	+	+	+/−
61 (24)	+	+	−	39 (17)	+	+	−
38 (4)	+	+/−	−	12 (9)	+	+/−	−
10 (0)	+	−	−	1 (2)	+/−	−	−
0	−	−	−	−	−	−	−
0	−	−	−	−	−	−	−

[a]Density of *R. solanacearum* in undiluted soil extracts as determined by IFC. Within brackets the standard deviations are given ($n = 3$). Non-target bacteria were present in densities of 2×10^4 cfu g^{-1} in sandy loam and in 10^5 cfu g^{-1} in clay soil.
[b]Presence of *R. solanacearum* on SMSA-plates was checked by testing colonies with a typical colony appearance with IFC and by IF on a 10-fold dilution of all bacteria harvested from a SMSA-plate.
[c]+ = all 3 replicates positive, +/− = 1 or 2 replicates positive, − = all replicates negative.

The detection level of the SMSA dilution plating method was close to that of IFC. However, saprophytic bacteria were often found on SMSA-plates with a colony-type very similar to that of *R. solanacearum* and characterisation of suspect colonies was required. Moreover, quantification could be hampered by the presence of large numbers of slime-producing saprophytic soil bacteria growing on SMSA. Finally, quantification could also be affected by the recovery of bacteria on SMSA, which was previously described as variable for race 3 strains (Engelbrecht, 1994).

The results with the tomato bioassay for detection in soil extracts were in agreement with the results of Elphinstone et al. (1996) for potato tuber macerates. Wilting was induced only at relative high pathogen populations ($>10^4$ cfu g^{-1} of soil) which makes this assay less suitable for detection of *R. solanacearum* in soil. The bioassay, however, may be used if the soil is suspected to harbour large numbers of cells in a 'viable but non culturable' (VBNC) state, which could be revived in the presence of host tissue. The presence of cells in a VBNC-state has been demonstrated for several soil bacterial species (Bloomfield et al., 1998; Oliver, 1995), and may play an important role in plant bacterial disease epidemiology.

In general, the physiological status of the bacterial pathogen, may influence detection by cultivation procedures. In this study, soils were sampled directly upon seeding with actively growing bacteria from a nutrient rich agar medium, while after persistence under conditions of low nutrient availability, such as in soil, cultivation on media with antibiotics may be impeded.

As a high proportion of strains (21 out of 30) which had been previously shown to cross-react with *R. solanacearum* antiserum in IF or ELISA (Griep et al., 1998), also cross-reacted when tested in IFC, the risks of false positive results would be high. Of the 21 strains which also cross-reacted in IFC, 3 were previously described as negative in both IF and ELISA, possibly because in IFC higher antibody

concentrations are used. Seven strains, cross-reacting in IFC, reacted exclusively in IF, which may be explained by cross-reacting cell wall bound antigens, not detected in ELISA. Four strains cross-reacting in IFC, reacted in ELISA, but not in IF. In this case soluble antigens may have been responsible, which are not seen in IF, but can be seen as a fluorescent halo in IFC.

Occasionally positive colonies from cross-reacting bacteria were also found in natural soils and although often these can be differentiated from *R. solanacearum* in IFC by colony appearance and staining intensity, confirmation procedures remain indispensable for reliable testing. This is particularly important when *R. solanacearum* is present in low numbers in the presence of cross-reacting bacteria. In order to avoid false-positive IFC-results, isolation of the bacteria by dilution plating followed by identification as well as PCR-amplification of test bacteria have each their own merits and drawbacks as was demonstrated previously for several plant associated bacteria (Van der Wolf et al., 1995, 1996). PCR-amplification, based on primers D2/B, allowed specific and rapid confirmation of *R. solanacearum* biovar 2 taken from IFC-positive colonies, although the efficiency was less than found for dilution plating on SMSA. The negative PCR-results could have been caused by the presence of PCR-inhibiting components, which could be diluted by testing lower concentrations of the test material or removed by DNA-purification methods. SMSA-dilution plating of sampled cells from colonies was shown to be highly efficient and is especially useful if pure culture identification is required. Dilution plating of cells from IFC-positive colonies on non-selective media can also be used for isolation and characterisation of cross-reacting bacteria. Isolation of cross-reacting bacteria can be important for further improvement of serological detection methods.

The risk for false-negative results, due to the serological variation is considered as low. The current polyclonal antibodies were found to react predominantly with the immunogenic lipopolysaccharide (LPS) O-chains on Western blots (unpublished results), which seem to be highly conserved in the biovar 2 group (Griep et al., 1998). Spontaneous avirulent mutants of *R. solanacearum*, which may be commonly present in soil, produce reduced amounts of extracellular polysaccharides (Hendrick and Sequeira, 1984), but still react strongly with the antibodies targeting the LPS O-chain (unpublished results).

The need to confirm positive IFC results could be averted in the future by using specific monoclonal antibodies instead of polyclonal antibodies. Two commercial sources are available now, one against extracellular polysaccharides of *R. solanacearum*, reacting with all biovars and the other, a recombinant antibody (scFv7) against the LPS of *R. solanacearum* showing a high specificity for biovar 2 strains (Alvarez and Benedict, 1990; Rabenstein et al., 1998; Griep et al., 1998). Their use in IFC is being evaluated currently using an indirect colony staining procedure.

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