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Sequence analysis and detection of *Ralstonia solanacearum* by multiplex PCR amplification of 16S–23S ribosomal intergenic spacer region with internal positive control

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Abstract

Polymerase chain reaction (PCR) methods for detection and differentiation of *Ralstonia solanacearum* strains were compared. The 16S–23S rRNA gene ITS sequence data revealed the two main sequence clusters (divisions I and II) of *R. solanacearum* and further subclusters of division II. Based on this sequence data, primers were designed which differentiated divisions I and II. Furthermore, to improve reliability of the PCR assay for routine detection of *R. solanacearum* in host plants, a novel multiplex PCR assay was developed in which the pathogen-specific sequences are coamplified with host plant DNA as an internal PCR control (IPC). The assay was validated during routine testing of potato samples submitted in official surveys. Of 4300 samples from 143 cultivars, 13 tested positive in both multiplex PCR and immunofluorescence (IF) assays and could be confirmed by bioassay in tomato seedlings and reisolation of the pathogen. The IPC was successfully amplified from all samples tested. A further 12 samples gave positive IF results which were not confirmed by either the multiplex PCR or tomato bioassay, indicating a greater specificity of the latter two assays.

Introduction

The gram-negative bacterium *Ralstonia solanacearum* (Smith) (Yabuuchi et al. 1995) (syn. *Burkholderia solanacearum* (Smith) (Yabuuchi et al., 1992); syn. *Pseudomonas solanacearum* (Smith) Smith) is the causal agent of bacterial wilt disease of hundreds of diverse plant species, including economically important crops such as potato and tomato (Hayward, 1991). *R. solanacearum* belongs to the beta subclass of Proteobacteria (Li et al., 1993) and is a heterogeneous species with significant phenotypic and genetic diversity (Cook et al., 1989; Hayward, 1991; Ito et al., 1996; Jaunet and Wang, 1999; Thwaites et al., 1999). DNA–DNA hybridisation (Roberts et al., 1990) and 16S rRNA sequence analysis (Li et al., 1993; Taghavi et al., 1996) revealed close genetic relationship of *R. solanacearum* with *Pseudomonas syzygii*, the causal agent of Sumatra disease of cloves (Roberts et al., 1990; Eden-Green, 1994) and the blood disease bacterium (BDB) of banana (Eden-Green and Sastraatmadja, 1990). *R. solanacearum* has been classified into races and biovars according to host range (Buddenhagen et al., 1962; He et al., 1983; Pegg and Moffett, 1971) and carbon utilisation (Hayward, 1964; Hayward, 1990) respectively. The two classifications are largely incompatible with overlapping races and biovars.

Based on restriction fragment length polymorphisms (RFLP; Cook et al., 1989) and 16S rRNA sequences (Li et al., 1993; Taghavi et al., 1996) *R. solanacearum* can be divided into two divisions: division I including

biovars 3, 4 and 5 and division II with biovars 1 and 2. On the basis of physiological properties and RFLP, the strains of biovar 2 were further subdivided into strains of biovar 2T, containing metabolically more versatile strains, and biovar 2A (synonymous with race 3), which is largely restricted to the narrow host range of the solanaceous crops potato, tomato, eggplant and related weeds (Cook and Sequeira, 1994; French, 1994).

Recent analyses by pulsed-field gel electrophoresis (Smith et al., 1995), repetitive sequence (rep)-PCR (Van der Wolf et al., 1998; Smith et al., 1998) and PCR-RFLP (Poussier et al., 1999) , determined genetic diversity and population structure of *R. solanacearum* in epidemiological studies and exhibited genetic homogeneity of biovar 2A strains. Originally regarded as a pathogen widely distributed in tropical and subtropical potato-growing regions worldwide, the economic importance of *R. solanacearum* biovar 2A increased recently in temperate Europe as a result of several outbreaks of potato brown rot (Olsson, 1976; Stead et al., 1996; Anonymous, 1997; Janse et al., 1998). The vascular pathogen *R. solanacearum* causes wilting of potato plants and rotting of tubers but also survives latently in potato tubers without causing symptoms (Ciampi et al., 1981). The main routes of transmission may be attributed to movement of latently infected seed potatoes (Janse, 1988) and to the irrigation of potatoes with contaminated surface water (Olsson, 1976; Elphinstone, 1996). Phytosanitary regulations have been introduced for the control and eradication of potato brown rot in Europe (Anonymous, 1998).

Methods currently recommended for the detection of latent *R. solanacearum* infection of potato (Anonymous, 1998) include indirect immunofluorescent antibody staining (IF assay; Janse, 1988), culture on semi-selective media (Elphinstone et al., 1996) and a tomato bioassay (Janse, 1988). Complimentary procedures include ELISA (Robinson-Smith et al., 1995), fluorescent *in situ* hybridisation (Wullings et al., 1998) and PCR assays (Seal et al., 1993, Opina et al., 1997; Fegan et al., 1998b; Boudazin et al., 1999; Pastrik and Maiss, 2000; Poussier and Luisettit, 2000). Serological techniques can lack specificity due to cross-reactions of polyclonal antibodies with other bacteria and may have limited sensitivity (Janse, 1988; Elphinstone et al., 1996). In contrast, assays using the polymerase chain reaction (PCR) offer potential advantages for rapid, highly specific and sensitive detection and identification of pathogenic bacteria. In routine assays, the interpretation of negative results can be difficult since these can be due either to the absence of the pathogen, failed DNA isolation or to inhibition of the PCR reaction by components of soil or plant extracts. In the latter two cases, false-negative results are obtained (Arulappan et al., 1996; Elphinstone et al., 1996). The sensitivity of *Taq* DNA polymerase to inhibition by factors present in biological samples is well known (Powell et al., 1994; Tsai and Olsen, 1992), and a number of chemicals used in DNA extraction procedures are known to interfere with DNA amplification (Rossen et al., 1992). The exclusion of false-negative results is essential where PCR-based tests are to be used as screening assays.

Multiplex PCR amplification (Chamberlain et al., 1991; Sandery et al., 1996) provides a means for reliable pathogen detection in routine testing by simultaneous amplification of more than one DNA region of interest in a single reaction mixture. The simultaneous amplification of an internal PCR control (IPC), which indicates both successful DNA isolation and potential presence of PCR inhibitors within the DNA extract, avoids the problem of false-negative PCR results (Pastrik, 2000) and is therefore recommended for the detection of *R. solanacearum* in potatoes (Elphinstone et al., 2000).

This study describes the sequence analysis of the intergenic spacer (ITS) in the rRNA operon between 16S and 23S rRNA genes of *R. solanacearum* strains representing members of biovars 1–5 and the BDB. This region of the genome is not subject to the same selective pressure as the rRNA structural genes and has been demonstrated to exhibit considerable variability for specific identification of bacteria (Barry et al., 1991; Jenson et al., 1993; Li and DeBoer, 1995; Scheinert et al., 1996; Pastrik and Rainey, 1999). Based on the sequence data, primers were selected for the specific identification of *R. solanacearum* divisions I and II. Both divisions have quarantine status in Europe but host ranges, risks and control strategies vary with the strain. Subsequently, to improve the reliability of the PCR assay for routine screening, a novel multiplex PCR assay was optimised for detection of *R. solanacearum* in host plants, involving coamplification of host DNA as an IPC.

Materials and methods

Bacterial strains and culture conditions

Bacteria (Tables 1 and 2) were obtained either from the Göttinger Collection of Phytopathogenic Bacteria

Table 1. Strains of *Ralstonia solanacearum* and the blood disease bacterium used in this study and accession numbers of sequences deposited in *EMBL*

Bacteria	Source code[1]	Alternative codes[2]	Biovar	Host	Origin	Accession number[3]
Ralstonia solanacearum	NCPPB 3970[4]	**R651**/CIP414	1 (Bugtok)	Banana	Philippines	AJ277768
	NCPPB 3971[4]	**R495**	1 (Bugtok)	Banana	Philippines	AJ277850
	NCPPB 2314[4]	**R133**	1 (Moko)	Plantain	Colombia	AJ277848
	NCPPB 4025[4]	**R283**/UW167	1 (Moko)	Banana	Costa Rica	AJ277777
	NCPPB 3969[4]	**R367**/CIP32	1 (Moko-SFR)	Plantain	Venezuela	AJ277849
	NCPPB 4027[4]	**R301**/UW26	1	Tomato	USA	AJ277769
	NCPPB 3976[4]	**R285**/UW256	1	Potato	Costa Rica	AJ277852
	NCPPB 1331		2A	Potato	India	AJ277770
	NCPPB 3980[4]	**R309**/CIP335	2A	Potato	Australia	AJ277767
	NCPPB 2505[4]		2A	Potato	Sweden	AJ277853
	NCPPB 3982[4]	**R576**/CIP238	2A	Potato	Chile	AJ277854
	NCPPB 4028	**R303**/UW19	2A	Potato	Colombia	
	EURS 1-70	(70 isolates)	2A	Potato	Europe	
	NCPPB 3989	**R568**/CIP226	2T	Potato	Brazil	
	NCPPB 3985[4]	**R578**/CIP312	2T	Eggplant	Peru	AJ277771
	NCPPB 3986[4]	**R583**/CIP172	2T	Potato	Peru	AJ277772
	NCPPB 3988[4]	**R780**	2T	Potato	Indonesia	AJ277855
	NCPPB 3992	**R278**/UW147	3	Tobacco	Australia	
	NCPPB 3996[4]	**R276**/UW130	3	Tomato	Peru	AJ277856
	NCPPB 3995[4]	**R304**/UW8	3	*E. odoratum*	Costa Rica	AJ277773
	NCPPB 4002	**R279**/UW151	4	Ginger	Australia	
	NCPPB 4003[4]	**R294**/UW359	4	Ginger	China	AJ277774
	NCPPB 4006	**R289**/UW378	4	Olive	China	
	NCPPB 3850[4]	**R288**/UW373	5	Mulberry	China	AJ277775
	NCPPB 3851[4]	**R292**/UW361	5	Mulberry	China	AJ277776
Blood disease bacterium	NCPPB 4026[4]	**R223**		Banana	Indonesia	AJ277851
	NCPPB 3726			Banana	Indonesia	

[1]Isolates obtained from the National Collection of Plant Pathogenic Bacteria (NCPPB), Central Science Laboratory, UK.
[2]Alternative codes from culture collections at IACR-Rothamsted, UK (R), International Potato Center, Peru (CIP) and University of Wisconsin, USA (UW).
[3]*EMBL* database accession number.
[4]Strains from which the intergenic spacer region between 16S and 23S rRNA genes was sequenced.

(GSPB; Göttingen, Germany), the German Collection of Microorganisms and Cell Cultures (DSMZ; Braunschweig, Germany), or from the National Collection of Plant Pathogenic Bacteria (NCPPB; Central Science Laboratory York, UK). *R. solanacearum* strains were grown on casamino peptone glucose agar (Kelman, 1954) at 28 °C. All *Clavibacter* spp. were grown on yeast extract glucose mineral salts agar (YGM; Anonymous, 1993) at 23 °C. Other bacteria were cultured on yeast peptone nutrient agar (YPN; Rhodes, 1959) at 24 °C. Identification of all *R. solanacearum* strains to biovar was confirmed using the methods of Hayward (1964) and Hayward et al. (1990).

Isolation of nucleic acids

DNA was extracted as described by Pastrik (2000). For the isolation of bacterial genomic DNA, a loopful of a bacterial culture was suspended in 1 ml PBS buffer (0.14 M NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, 1.8 mM KH_2PO_4; pH 7.4) and centrifuged for 2 min at 13,000 × *g* and 4 °C. The pellet was resuspended in 320 µl lysis-buffer (100 mM NaCl; 10 mM Tris–HCl, pH 8.0; 1 mM EDTA, pH 8.0), placed on a heating block at 95 °C for 10 min and cooled on ice for 5 min. Then, 80 µl lysozyme stock solution (50 mg/ml lysozyme in 10 mM Tris–HCl, pH 8.0) was added, and the sample was incubated for 30 min at 37 °C. The DNA was purified using the Easy-DNA-Extraction-kit (Invitrogen, De Schelp, the Netherlands). Solution A (220 µl) was added and the mixture was incubated for 30 min at 65 °C. After addition of 100 µl solution B and mixing, 500 µl chloroform was added and the mixture was centrifuged for 20 min at 20,000 × *g*. The aqueous phase was transferred to a new tube and DNA was precipitated by addition of ethanol to 70%, and the

Table 2. Bacterial strains used in this study

Bacteria	Culture code	Bacteria	Culture code
Ralstonia eutrophus	DSM 531^T	*Clavibacter michiganensis*	DSM 20134
	NCPPB 4048	subsp. *michiganensis*	DSM 46364^T
	NCPPB 4049		GSPB 382
			GSPB 390
Ralstonia pickettii	DSM 6297^T		
	NCPPB 4075	*Clavibacter michiganensis*	DSM 20157^T
	NCPPB 4076	subsp. *insidiosus*	GSPB 2225
			GSPB 29
Pseudomonas syzygii	NCPPB 3790		
	NCPPB 3791	*Clavibacter michiganensis*	DSM 20741^T
		subsp. *tesselarius*	
Burkholderia andropogonis	DSM 9511^T		
	NCPPB 1127	*Clavibacter michiganensis*	DSM 20400
	NCPPB 2869	subsp. *nebraskensis*	DSM 20401
			DSM 7483^T
Burkholderia caryophylli	DSM 50341^T		
	NCPPB 353	*Erwinia carotovora*	DSM 60424
	NCPPB 2151	subsp. *atroseptica*	GSPB 401
Burkholderia cepacia	DSM 7288^T	*Erwinia carotovora*	DSM 30168^T
	NCPPB 4074	subsp. *carotovora*	GSPB 133
	NCPPB 945		
	NCPPB 946	*Erwinia chrysanthemi*	GSPB 421
			DSM 30177
Burkholderia gladioli	DSM 4285^T		
		Erwinia rhapontici	GSPB 454
			GSPB 455
Burkholderia glumae	DSM 9512^T		
	NCPPB 3708	*Pseudomonas syringae*	GSPB 1440
		pv. *atrofaciens*	GSPB 1392
Burkholderia plantarii	NCPPB 3590		
	DSM 9510^T	*Pseudomonas syringae*	DSM 50302
		pv. *morsprunorm*	
Clavibacter michiganensis	DSM 46300		
subsp. *sepedonicus*	GSPB 1522	*Pseudomonas syringae*	GSPB 1495
	GSPB 2238	pv. *phaseolicola*	GSPB 567
	GSPB 2249		

resulting pellet was washed with 80% ethanol. After the final centrifugation the DNA was resuspended in 100 µl of sterile ultra pure water. Nucleic acid concentration was estimated from the intensity of ethidium bromide fluorescence (Sambrook et al., 1989) by using 2D-Densitometry-Software (Cybertech, Berlin, Germany) and DNA molecular weight marker I (Boehringer Mannheim, Germany) as a standard DNA.

For the isolation of DNA from potato samples, potato macerate (100 µl, prepared as outlined below) was mixed with 220 µl lysis buffer (100 mM NaCl; 10 mM Tris–HCl, pH 8.0; 1 mM EDTA, pH 8.0) and further DNA purification was performed by the procedure described above.

Primers

Primers employed in this study (Table 3) were HPLC-purified and purchased from Life Technologies (Germany). For the IPC, amplification of the primer set NS-5-F/NS-6-R (Dams et al., 1988; White et al., 1990) was employed. These primers are based on conserved nucleotide sequences from 18S rRNA genes from *Saccharomyces cerevisiae, Dictyostelium*

Table 3. Primer sequences and sizes of amplified DNA-fragments

Primer	Primer specificity	Primer sequences 5′-3′	Size of PCR product (bp)
RS-1-F (forward)	All *R. solanacearum*	ACT AAC GAA GCA GAG ATG CAT TA	
RS-1-R (reverse)	Division 2 *R. solanacearum*	CCC AGT CAC GGC AGA GAC T	718
RS-3-R (reverse)	Division 1 *R. solanacearum*	TTC ACG GCA AGA TCG CTC	716
NS-5-F (forward)*	Eucaryotic DNA	AAC TTA AAG GAA TTG ACG GAA G	310
NS-6-R (reverse)*	Eucaryotic DNA	GCA TCA CAG ACC TGT TAT TGC CTC	

*Dams et al., 1988; White et al., 1990.

discoideum and *Stylonicha pustulata* (Dams et al., 1988) and they amplify a DNA fragment of 310 bp from *R. solanacearum* host plants, including potato, eggplant and tomato.

Sequence analysis of the ITS region between 16S and 23S rRNA genes

For amplification of the intergenic spacer region between 16S and 23S rRNA genes the eubacterial universal primers 1493 forward (16S rDNA; 5′-AGTCGTAACAAGGTAGCCGT-3′) and 23 reverse (23S rDNA; 5′-GTGCCAAGGCATCCACC-3′) were used (Li and DeBoer, 1995). The reaction mixture contained 1× reaction-buffer (10 mM Tris–HCl, 1.5 mM $MgCl_2$, 50 mM KCl; pH 8.4), 100 µM of each dNTP (Boehringer Mannheim, Germany), 0.2 µM of each primer and 1 ng DNA in 100 µl reaction volume overlaid with one drop of mineral oil. After denaturation for 3 min at 94 °C, 2 U of *Taq* DNA polymerase (Boehringer Mannheim, Germany) was added and the reaction mixture was submitted to 25 cycles of 94 °C for 30 s, 48 °C for 30 s and 72 °C for 60 s with a final extension of 72 °C for 10 min. The PCR products were purified using a Prep-a-Gene Kit (Biorad, USA) according to the manufacturer's instructions. The purified PCR products were used as template for sequencing reactions. Sequencing reactions were carried out using a *Taq* DyeDeoxy™ Terminator Cycle Sequencing Kit (Applied Biosystems, USA) according to the manufacturer's instructions using a Perkin Elmer 9600 thermocycler. The sequence reactions were electrophoresed using the Applied Biosystems 373A DNA Sequencer.

Amplification with division-specific primers and multiplex PCR

Primers selected for specific amplification were HPLC-purified and purchased from Life Technologies (Germany). PCR was performed in a MJ Research thermocycler (PTC 200). The 25-µl PCR reaction mixture contained 1× reaction buffer (20 mM Tris–HCl, pH 8.4; 50 mM KCl), 1.5 mM $MgCl_2$, 100 µM of each dNTP (Boehringer Mannheim, Germany), 0.8 µM each of primers RS-1-F/RS-1-R or 0.4 µM each of primers RS-1-F/RS-3-R or additionally in multiplex PCR 0.06 µM each of primers NS-5-F/NS-6R, 1 U *Taq* DNA polymerase (Life Technologies, Germany) and 1 ng of bacterial genomic DNA or 5 µl of the DNA solution from potato samples. The following PCR conditions were used: Initial denaturation at 95 °C for 5 min, followed by 35 reaction cycles of 95 °C for 30 s, 58 °C for 30 s and 72 °C for 45 s. After the final reaction cycle the mixture was held at 72 °C for 5 min and stored at 4 °C. After the PCR, 12 µl aliquots of the reaction mixture were resolved by electrophoresis in a 2% agarose gel and DNA fragments were visualised by staining in a solution of 0.5 µg/ml ethidium bromide.

Potato samples

Composite samples of 200 seed potato tubers were submitted to Pflanzenschutzamt Hannover or to Institut für Pflanzenbau und Pflanzenschutz, Oldenburg (IPP) for official surveys of potatoes and were tested for *R. solanacearum* using the immunofluorescence (IF)-assay and the designed multiplex PCR assay. The IF-test was performed according to the

standard EC-protocol for the detection and identification of *R. solanacearum* in samples of potato tubers (Anonymous, 1998) using a polyclonal rabbit antiserum (Adgen-100302). Approximately 4300 composite samples of 143 different cultivars (as listed in Pastrik, 2000) were tested. Sample preparation was performed by the procedure described below.

Sample preparation of potato macerates

In accordance with the official EC method for detection and diagnosis of *R. solanacearum* in potatoes (Anonymous, 1998) composite samples each consisting of 200 potato tubers were processed as described by Pastrik (2000). Potato tubers were washed in tap water to free them from adherent soil. A small core of tissue (containing vascular tissue) was removed from the stolon end of each tuber and the cores were combined in a plastic bag. Molecular grade sterile double-distilled water (30 ml) was added, and the potato tissue was thoroughly macerated (2–3 min) using a 'Homex' apparatus (Bioreba, Switzerland). The macerate was filtered through a 60–90 μm column filtration system (Macherey-Nagel, Germany) and collected in a centrifuge tube. The filtrate was centrifuged at 10,000 × *g* for 10 min and the resulting pellet was resuspended in 1 ml sterile ultra pure water.

Sensitivity of PCR

To determine the detection limit of the PCR, a culture of *R. solanacearum* (NCPPB 1331; approximately 10^8 colony-forming units (cfu) per ml) was serially diluted by 10-fold increments in sterile water. Aliquots (100 μl) of the serial dilutions were added into plastic bags containing tissue samples (200 tissue cores) of healthy potatoes. Samples were macerated and treated as described above. Concentrations of viable bacteria were estimated as the number of colonies which developed after plating 100 μl of the serial dilutions on casamino peptone glucose agar (Kelman, 1954).

Restriction analysis

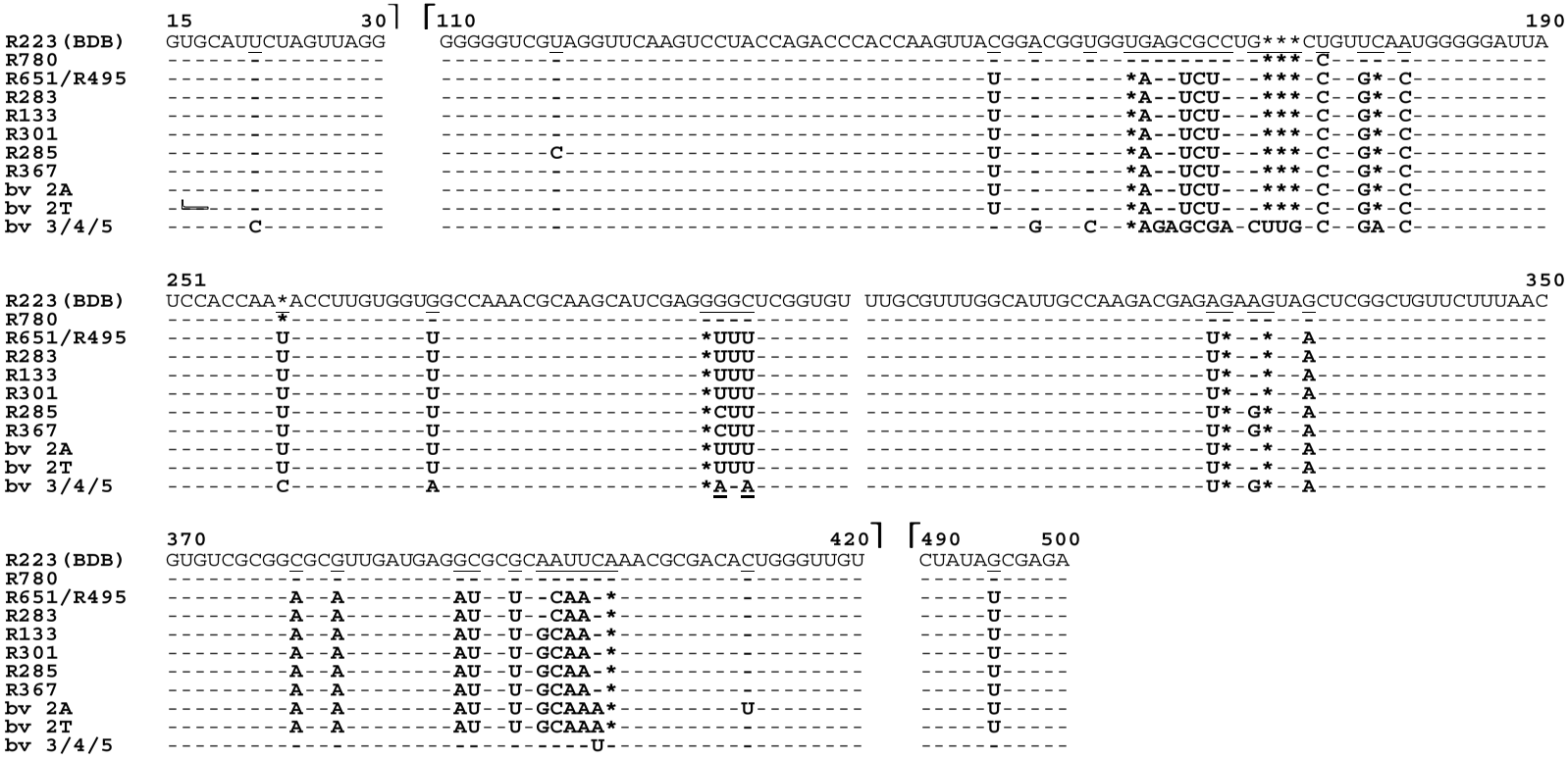
The specificity of the amplified PCR products was confirmed by restriction analysis with *Bsm* I. Samples of 10 μl of PCR product were digested with 5 U of *Bsm* I (Boehringer Mannheim, Germany) at 65 °C for 45 min. Restriction products were analysed by electrophoresis on a 2% agarose gel and visualised by staining with 0.5 μg ml^{-1} ethidium bromide.

Results

Sequence analysis of the ITS spacer region

The eubacterial universal primers 1493 forward and 23 reverse amplified a DNA-fragment of approximately 590 bp in all strains of *R. solanacearum* and the BDB. The complete spacer regions between the 16S and 23S rRNA genes of strains representing all biovars were sequenced and manually aligned, for 19 strains of *R. solanacearum*, isolated from different host plants and locations, and one BDB strain. The sequence data were deposited at EMBL-European Bioinformatics Institute (Cambridge, UK), accession numbers for *R. solanacearum* strains are given in Table 1. From seven *R. solanacearum* biovar 1 strains tested, strains R283 (isolated from banana with moko disease in Costa Rica), R495 and R651 (both isolated from banana with bugtok disease in the Philippines), were found to be identical in the nucleotide sequence of the ITS, but different at one nucleotide position to strains R133 (isolated from plantain with moko disease in Colombia) and R301 (tomato, USA) and different at two nucleotide positions to strains R285 (potato, Costa Rica) and R367 (Moko-SFR (small, fluidal, round strain) from plantain, Venezuela) (Table 4). Furthermore, sequences of strains R285 and R367 were found to be different at one nucleotide position compared to each other. While the ITS sequences of *R. solanacearum* strains causing Moko (R283) and Bugtok (R651 and R495) disease of banana showed 100% similarity, the sequences of BDB strain R223, which causes blood disease of banana in Indonesia, had only a low level of similarity to other *R. solanacearum* strains sequenced with the exception of strain R780 an atypical biovar 2T strain isolated from potato in Indonesia. Sequences of both strains differed at only one nucleotide position. The strains of *R. solanacearum* biovars 2A and 2T (R578, R583) indicated high genetic relatedness and were distinguishable only at one nucleotide position. The biovar 2A and 2T strains sequenced clustered with the biovar 1 strains sequenced. All strains representing biovars 3, 4 and 5 had identical sequences. There were multiple nucleotide differences between strains representing these two groups of biovars (Table 4).

Table 4. Partial sequence alignment with differences in base composition of the ITS of the BDB, *R. solanacearum* strains and biovars. Dashes indicate identical base composition compared to the sequence of BDB. Asterisks indicate sequence gaps



Amplification with biovar-specific primers

PCR primers were selected on the basis of 16S rRNA gene and intergenic spacer region sequences specific to divisions I and II of *R. solanacearum*. The primer pairs consisted of a forward primer, which was universal for all *R. solanacearum* strains and reverse primers specific for each division (Table 3). For the identification and detection of division I-strains (biovars 3, 4 and 5) the primers Rs-1-F/Rs-3-R were used and for the identification and detection of division II-strains (biovars 1, 2A and 2T) the primers Rs-1-F/Rs-1-R were employed. To test the specificity of the primer pairs, amplification was carried out with genomic DNA of the bacterial strains listed in Tables 1 and 2. The primer pairs amplified a specific DNA-fragment only from DNA of the *R. solanacearum* division they were designed for. Amplification products were not obtained with either primer pair from DNA of *R. solanacearum* strain R780, BDB and *P. syzygii*, or other bacteria tested.

Multiplex PCR for the detection of R. solanacearum *in potato tubers*

Primer sets Rs-1-F/Rs-1-R (*R. solanacearum* division II-specific) and NS-5-F/NS-6-R (plant-specific, internal PCR-control) were used simultaneously in the same reaction mixture. Optimisation experiments were performed with DNA extracts from potato tuber samples artificially inoculated with a serially diluted suspensions of *R. solanacearum* biovar 2A (NCPPB 1331). The reaction conditions of the multiplex PCR were carefully optimised as described by Pastrik (2000) with respect to concentrations of the primer sets, yield of PCR products and subsequent sensitivity of detection of *R. solanacearum*. The detection sensitivity of the multiplex PCR was compared with that obtained using only the primer set Rs-1-F/Rs-1-R in a PCR assay (Figure 1a). The highest sensitivity was achieved with 0.8 μM of primers Rs-1-F/Rs-1-R and 0.06 μM of primers NS-5-F/NS-6-R. Using higher concentrations of primer set NS-5-F/NS-6-R resulted in increased amplification of the plant-specific DNA fragment, but decreased sensitivity of detection of *R. solanacearum* (data not shown).

Using the optimised multiplex PCR protocol, it was possible to detect *R. solanacearum* added to potato core suspension in the range of 5–50 cfu per PCR reaction mixture (50–500 cfu per ml concentrated potato core suspension) (Figure 1b, lanes 6 and 7). This was

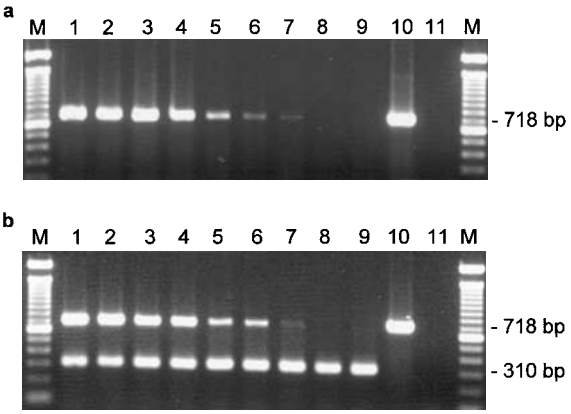


Figure 1. Sensitivity of PCR employing primer pair Rs-1-F/Rs-1-R (a) and multiplex PCR employing primer pairs Rs-1-F/Rs-1-R and NS-5-F/NS-6-R (b) for *R. solanacearum* in potato core fluid. Healthy potato tuber extracts were mixed with 10-fold dilutions of *R. solanacearum* cells (NCPPB 1331). Lanes 1–8, serial 1 in 10 dilutions of *R. solanacearum* cells ranging from 5×10^6 to 0.5 CFU per PCR reaction mixture; lane 9, healthy potato sample; lane 10, genomic DNA of *R. solanacearum* (NCPPB 1331); lane 11, negative control; lanes M, DNA size marker (100 bp ladder, Life Technologies, Germany).

equivalent to the detection sensitivity using the PCR assay employing only the primer set Rs-1-F/Rs-1-R (Figure 1a). Multiplex PCR with DNA extracts from potato tubers infected with *R. solanacearum* generated 2 discrete DNA fragments in the size of 718 bp (*R. solanacearum*-specific) and 310 bp (plant-specific) (Figure 1b, lanes 1–7). With increasing numbers of the bacterium the *R. solanacearum*-specific PCR product was detected with stronger signal intensity, but amplification of the plant-specific DNA fragment decreased. Only the plant-specific fragment of the size of 310 bp was amplified from samples of potato core suspension without *R. solanacearum* (Figure 1b, lane 9). In the multiplex PCR, only the *R. solanacearum*-specific DNA fragment and no plant-specific DNA fragment was amplified from genomic DNA extracts of *R. solanacearum* (Figure 1b, lane 10).

The specificity of the multiplex PCR was tested again by amplification of genomic DNA from the bacterial strains listed in Tables 1 and 2, potato DNA and seedlings of tomato (cv. Moneymaker) and eggplant (cv. Black Beauty).

The *R. solanacearum*-specific DNA fragment was specifically obtained from division II strains (biovar 1, 2A and 2T) including 70 strains of *R. solanacearum* isolated from potato in Europe but not from *R. solanacearum*-strain R780 (biovar 2T from

Indonesia), BDB or bacteria of other genera tested (data not shown). Amplification of potato, tomato and eggplant DNA in the multiplex PCR generated a 'universal' amplicon of 310 bp (data only shown for potato, Figure 2). The specificity of the amplified PCR products were confirmed by restriction digestion with *Bsm* I. The restriction site of *Bsm* I is present in the division 2-specific amplicon (primers Rs-1-F/Rs-1-R) but not in the division 1-specific PCR product amplified with primers Rs-1-F/Rs-3-R nor in the plant-specific DNA-fragment (Figure 3).

The suitability of the multiplex PCR assay for routine screening of potato samples for the presence of *R. solanacearum* was examined with approximately 4300 composite potato tuber samples of 143 different cultivars using parallel IF and multiplex PCR assays. Representative multiplex PCR results in surveys for *R. solanacearum* in potato seed samples were shown in Figure 2.

Of the 4300 composite samples, thirteen samples tested positive in both multiplex PCR and IF tests and subsequent bioassay on tomato. A further twelve samples tested positive in the IF assay, but negative in multiplex PCR and negative in the bioassay. Among 143 different potato cultivars tested, all amplified the expected universal plant-specific PCR product of 310 bp (data not shown).

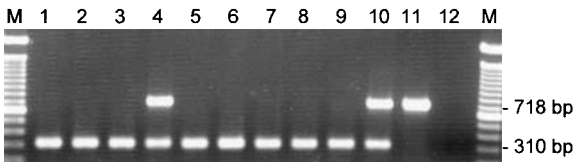


Figure 2. Representative multiplex PCR results in surveys for *R. solanacearum* in potato seed samples. Lanes 1–8, DNA of various potato seed samples; lane 9, DNA of potato extracts mixed with *C. michiganensis* subsp. *sepedonicus* cells (2×10^5); lane 10, DNA of potato extracts mixed with *R. solanacearum* (NCPPB 1331) cells (2×10^5); lane 11, genomic DNA of *R. solanacearum* (NCPPB 1331); lane 12, negative control; lanes M, DNA size marker (100 bp ladder, Life Technologies, Germany).

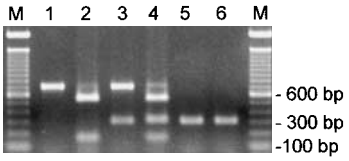


Figure 3. Restriction analysis of multiplex PCR products digested with *Bsm* I (Boehringer Mannheim, Germany). Lanes 1 and 2, undigested and digested *R. solanacearum* division 2-specific PCR products respectively; lanes 3 and 4, undigested and digested multiplex PCR products of a *R. solanacearum*-positive potato sample; lanes 5 and 6, undigested and digested plant-specific universal PCR product; lanes M, DNA size marker (100 bp ladder, Life Technologies, Germany).

Discussion

The heterogeneous species *R. solanacearum* represents an important plant pathogen worldwide. For the development of appropriate control measures the genetic diversity of the pathogen should be known. The comparison of ITS sequence similarity revealed, in general, two main clusters in accordance with the results of Cook et al. (1989), based on RFLP data and of Li et al. (1993) and Taghavi et al. (1996), based on 16S rRNA gene sequences. The ITS sequences were identical for all members of division I (biovars 3, 4 and 5). However, further subdivision within members of division II (biovars 1, 2A and 2T) was indicated by minor ITS sequence variations.

ITS sequences for the Moko strain R283 from Costa Rica and the Bugtok strains R651 and R495, both from the Philippines were identical. The sequences were distinct from those of the BDB strain, strain R780 and two other subclusters, one containing strains R301 (from tomato in the USA) and R133 (from plantain with Moko disease in Colombia), the other including strains R367 (from plantain in Venezuela) and R285 (from potato in Costa Rica). These observations supported the hypothesis of Eden-Green (1994), that strains causing Moko and Bugtok disease on banana in the Philippines may be the same organism, but different from those causing banana blood disease in Indonesia. The ITS data showed a sequence analogy of the BDB strain R223 with the biovar 2T strain R780 (isolated from potato in Indonesia) confirming the findings of Seal et al. (1999) and Taghavi et al. (1996) based on 16S rDNA sequencing and Fegan et al. (1998a) based on unpublished 16S–23S rRNA gene intergenic spacer region, as well as polygalacturonase and endoglucanase gene sequences. The authors suggested little genetic divergence of Indonesian *R. solanacearum* strains of BDB and *P. syzygii*, which have only been found in Indonesia, resulting in a separate cluster of strains within the *R. solanacearum* species complex. Although the strains R133 and R367 were both isolated from plantain they were isolated from different locations and their ITS sequences were distinct. The ITS data also indicated a close genomic

relationship between strains of *R. solanacearum* biovars 2A and 2T, but also their genetic distinction as previously described by Cook and Sequeira (1994) and French (1994).

Specific PCR assays developed for the identification of the *R. solanacearum* divisions I and II, allowed accurate classification of all *R. solanacearum* strains tested. No amplification product was produced by the BDB, *P. syzygii*, or the atypical biovar 2T strain R780, as has been described for other *R. solanacearum* PCR assays (Boudazin et al., 1999; Seal et al., 1999; Poussier and Luisetti, 2000).

For detection of *R. solanacearum* in potato tubers, a multiplex PCR assay combined highly specific and sensitive detection of the pathogen with coamplification of universal host-specific DNA as an internal PCR control (IPC) in one reaction mixture. A multiplex '*Taq*Man' PCR method for specific detection of *R. solanacearum* biovar 2A in potato tuber extracts was described recently by Weller et al. (2000). The conventional multiplex PCR assay described in this paper provides a simple lower cost alternative to the '*Taq*Man' method. Multiplex PCR assays employing an IPC have the advantage that, potentially false-negative PCR results, generated by unsuccessful DNA isolation or reaction failure of the PCR, can be monitored during the assay. As described by Pastrik (2000) the yield of the different PCR products and relative concentrations of the primer sets were both critical for maximum sensitivity of the *R. solanacearum* PCR assay. The detection level of the optimised multiplex PCR was equivalent to that found in PCR protocols employing a single primer pair designed to produce a single amplicon (Seal et al., 1993; 1999; Boudazin et al., 1999; Pastrik and Maiss, 2000; Poussier and Luisetti, 2000).

The multiplex PCR designed here has the advantage, that the reliability of the PCR assay was improved for routine screening by the amplification of the internal positive control (IPC). It is probable that for equivalent detection sensitivity under varying laboratory conditions additional standardisation of sample preparation and optimisation of laboratory-specific reaction conditions will be required.

Parallel IF and multiplex PCR assays on tuber extracts obtained during official potato surveys showed a good correlation between results of both tests. Out of 4300 composite samples, thirteen samples tested positive in both tests and confirmatory tomato bioassay (Janse, 1988). However, the increased specificity of the multiplex PCR was indicated in the case of 12 samples which tested positive in IF but negative in both the multiplex PCR and bioassay, probably as a result of non-specific cross-reactions with the polyclonal antibodies (Janse, 1988; Elphinstone et al., 1996). The universality of the IPC was demonstrated by the amplification of the plant-specific PCR product with all 143 different potato cultivars tested.

In conclusion, the methods described in this paper, which have been subsequently ring-tested in 19 EC quarantine testing laboratories, can permit rapid and reliable means for the identification, differentiation and detection of *R. solanacearum* and should provide effective alternative methods to the conventional tests used.

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