European Journal of Plant Pathology **106:** 255–265, 2000.

Specific detection of biovars of *Ralstonia solanacearum* in plant tissues by Nested-PCR-RFLP

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Accepted 7 December 1999

Key words: bacterial wilt, biovars discrimination, diagnosis, *hrp*

Abstract

A sensitive and specific assay, based on a Nested-PCR-RFLP protocol, was developed for the detection of biovars of *Ralstonia solanacearum*, the causal agent of bacterial wilt. Oligonucleotide primer pairs were selected within the *hrp* gene region. Specific amplification of the *hrp* fragments was obtained for all *R. solanacearum* strains and also for two closely related species, *Pseudomonas syzygii* and the blood disease bacterium. No amplification was observed for a wide range of other bacterial species, including *R. pickettii* and *Burkholderia cepacia*. Digestion with *Hin*dII provided four distinct restriction profiles specific to biovars or groups of biovars of *R. solanacearum*: one for biovar 1 strains originating from the Southern part of Africa, one for American biovar 1 and biovars 2 and N2 strains, one for biovars 3 and 4 strains, and one for biovar 5 strains. When applied to either pure culture or infected plant tissues, Nested-PCR allowed detection as low as 10^3 cfu ml^{-1}, which corresponds to 1 cfu per reaction. Amplification was partially or completely inhibited by compounds contained in plant extracts (potato plant and potato tuber, tomato, tobacco, eggplant, pepper and *Pelargonium asperum*). A combined PVPP/BSA treatment prior to amplification permitted reliable Nested-PCR detection of *R. solanacearum* strains in plant samples. Nested-PCR-RFLP, assessed with isolates from Reunion Island but also applicable to any *R. solanacearum* strain, provides a wide range of possible uses for identification, detection and epidemiological investigations.

Introduction

Ralstonia solanacearum, causal agent of bacterial wilt, is responsible for severe losses to many important crops in tropical, subtropical and warm temperate regions of the world (Hayward, 1991). Recently, several outbreaks of the disease were reported in Western Europe (Stead et al., 1996). *R. solanacearum* appeared to be rather heterogeneous and has been subdivided into five races based on host range (Buddenhagen et al., 1962; He et al., 1983; Pegg and Moffett, 1971) and into six biovars based on differences in the utilization of three disaccharides and three sugar alcohols (Hayward, 1964; Hayward et al., 1990; He et al., 1983). Furthermore, based on restriction fragment length polymorphism (RFLP) (Cook et al., 1989, 1991; Cook and Sequeira, 1994) and 16S rRNA gene sequences (Li et al., 1993; Taghavi et al., 1996; Seal et al., 1999), *R. solanacearum* species was divided into two major clusters related to geographical (Asian and American) origin of strains.

The biovar determination procedure, as described by Hayward (1964), requires a previous purification of the bacterium and an incubation period of more than 2 weeks. New molecular tools such as the PCR technique, which do not need previous cultivation of bacterium, have been successfully applied to the direct detection of many plant-pathogenic bacteria (Hartung et al., 1996; Hu et al., 1995; Leite et al., 1995; Maes et al., 1996; Minsavage et al., 1994; Roberts et al.,

1996). To discriminate biovars of *R. solanacearum*, Seal et al. (1992a) have developed tRNA consensus primers, but since similar PCR patterns were obtained with strains belonging to other plant bacterial species these primers could not be used for effective detection of *R. solanacearum* biovars.

A PCR-RFLP method has previously been used to assess the genetic diversity within a worldwide collection of *R. solanacearum* (Poussier et al., 1999). The whole *hrp* gene region, which is involved in the hypersensitive reaction and pathogenicity (Arlat et al., 1992; Boucher et al., 1992), was explored and this led to an accurate distinction of the major biovars of *R. solanacearum* and a clear discrimination between African biovar 1 strains and American biovar 1 strains.

The objective of this study was to exploit these results to develop a specific and sensitive procedure (Nested-PCR-RFLP) to identify and detect specific biovars of *R. solanacearum* in plant tissues. Most of these experiments were performed with isolates from Reunion Island. Reunion Island, where the disease has been recorded on 23 species (mainly potato, tomato, tobacco, eggplant, pepper and *Pelargonium asperum*) of plants from 12 families (Girard et al., 1993; Nicole, 1995) and where two races (1 and 3) and three biovars (1, 2 and 3) of *R. solanacearum* have been identified, is an excellent geographical area to check the usefulness of molecular tools.

Materials and methods

Bacterial strains and culture conditions

R. solanacearum strains used in this study were previously described (Poussier et al., 1999). Additional strains belonging to biovars N2 and 5 are listed in Table 1. The strains were grown on a modified Granada and Sequeira medium (Granada and Sequeira, 1983) (tryptone, 1 g l^{-1}; peptone, 10 g l^{-1}; agar, 18 g l^{-1}; glycerine, 6.3 ml l^{-1}; crystal violet, 0.002 g l^{-1}; polymyxine sulphate, 0.01 g l^{-1}; tyrothricine, 0.02 g l^{-1}; chloramphenicol, 0.005 g l^{-1}; triphenyltetrazolium chloride, 0.025 g l^{-1}; propiconazole, 0.4 ml l^{-1}; penicillin, 20 U l^{-1}; pH 7.2). Typically fluidal *R. solanacearum* colonies with characteristic tetrazolium red coloration (Kelman, 1954), were observed after 3 days incubation at 28 °C. To check the specificity of our molecular tools, other strains belonging to species more or less closely related to *R. solanacearum* (Table 1) were included and cultured on YPGA medium (yeast extract, 7 g l^{-1}; peptone, 7 g l^{-1}; glucose, 7 g l^{-1}; agar, 15 g l^{-1}; pH 7.2). Long-term storage of strains was achieved at −80 °C on beads in cryovials (Microbank™ Prolab Diagnostics, Austin, Texas, USA).

Primers

All primers were selected from the nucleotide sequence of the *hrp* genes cluster of the strain GMI1000 (accession number: Z14056; EMBL/GenBank/DDBJ databases) of *R. solanacearum*. Primers RS30 and RS31 (Table 2) used for the first-round PCR, delineated a 1993 bp fragment located over the *hrp*O and *hrp*N genes of the *hrp* gene region of *R. solanacearum*. For Nested-PCR (N-PCR), two additional sets of primers were selected within the fragment delineated by primers RS30 and RS31. These primer pairs (RS30a–RS31a and RS30b–RS31b) (Table 2) directed the amplification of a 256 bp and a 533 bp fragment respectively (Figure 1). Since we have designed two primer pairs, the test was based on multiplex PCR. All primers were designed by the OLIGO 5.0 software (National Biosciences, 1996) and synthesized by Genosys Biotechnologies, Cambridge, England.

PCR amplification, nested amplification and electrophoresis

Template DNA was prepared from either purified total bacterial DNA (Ausubel et al., 1991), or from a boiled (for 5 min) suspension of pure culture or infected plant extract. First stage PCR was performed with a 50 µl reaction mixture containing 1× reaction buffer 3 (supplied by the manufacturer with enzyme), 0.5 µM of primers (RS30 and RS31), 0.25 mM of each dNTP (Boehringer Mannheim, Meylan, France), 0.7 U of a mix (*Taq* and *Pwo*) of DNA polymerases (Expand™ Long Template PCR System; Boehringer Mannheim), water (HPLC grade; Sigma-Aldrich, Steinheim, Germany) and 1 µl of template DNA. The following thermal profile was used: initial denaturation (95 °C for 5 min) followed by 30 repeated cycles of denaturation (95 °C for 30 s), annealing (64 °C for 30 s), extension (68 °C for 2 min for the first 10 cycles and addition of 20 s for each new cycle for the last 20 cycles), and final extension (68 °C for 7 min). For the second stage (N-PCR), 1 µl from the first-round PCR product was added as template DNA to 49 µl of a reaction mixture which contained 1× reaction buffer

Table 1. R. solanacearum strains and other bacterial strains used in this study and not previously described (Poussier et al., 1999)

Organism	Biovar	Strain	Other designation	Source[1]
Ralstonia solanacearum	N2	JT653	UW470	D
	N2	JT654	UW477	D
	N2	JT658	R361	E
	N2	JT686	R572	E
	N2	JT676	NCPPB3990	C
	N2	JT677	NCPPB3987	C
	5	JT661	R292	E
	5	JT685	R322	E
Erwinia chrysanthemi		JT544		A
Xanthomonas sp. pv. *mangiferae-indicae*		JT545		A
X. vesicatoria		JT546		A
Pseudomonas fluorescens		JT547		A
P. fuscovaginae		JT548		A
P. cichorii		JT549		A
P. syringae pv. *tomato*		JT550		A
Clavibacter michiganensis subsp. *michiganensis*		JT551		A
Ralstonia pickettii		JR660	CFBP2459	B
		JR665.1		A
		JR665.2		A
R. eutropha		JS755	GMI8105	F
Burkholderia cepacia		JR661	CFBP2227	B
		JR664.1		A
		JR664.2		A
P. syzygii		JV1010	R024	E
		JV1011	R028	E
Blood disease bacterium		JT656	R604	E
		JT657	R230	E
		JT680	NCPPB3726	C

[1]Strains were contributed by: A, Laboratoire de phytopathologie, CIRAD-FLHOR, Ligne Paradis, 97448 Saint-Pierre, Réunion, France; B, Collection Française de Bactéries Phytopathogènes, Angers, France; C, National Collection of Plant Pathogenic Bacteria, Harpenden, United Kingdom; D, D. Cook and L. Sequeira, Department of Plant Pathology, University of Wisconsin-Madison, USA; E, Institute of Arable Crops Research-Rothamsted, Harpenden, United Kingdom; F, M. Arlat and P. Barberis, CNRS-INRA, Auzeville, Castanet-Tolosan Cedex, France.

Table 2. Primers selected from the *hrp* gene sequence of the strain GMI1000

Primer designation	Sequence (5′–3′)
RS30	GAAGAGGAACGACGGAAAGC
RS31	CGAACAGCCCACAGACAAGA
RS30a	GGCGCTGGCGGTGAACATGG
RS31a	CAACATCCTGGCCGGCATCGTG
RS30b	TCTTGCGCTCGCCCTTGATGTG
RS31b	CGACAGCAGCCGGCACCC

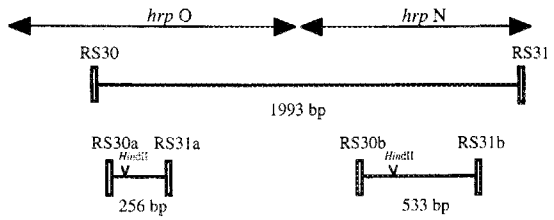

Figure 1. Nested-PCR-RFLP: position of the first- and second-round PCR primer pairs within the *hrp* gene region, size of resulting amplified fragments, and location of *Hin*dIII restriction sites (presence or absence are specified in the text).

(supplied by the manufacturer with enzyme), 1.5 mM of $MgCl_2$, 0.3 mM of each dNTP, 0.35 µM of each primer (RS30a–RS31a and RS30b–RS31b) and 1 U of *Taq* DNA polymerase (GIBCO BRL, Life Technologies, Cergy Pontoise, France). The amplification procedure included an initial denaturation at 96 °C for 2 min followed by 20 cycles of 94 °C for 20 s, 70 °C for 30 s, 72 °C for 30 s and final extension at 72 °C for 10 min.

All the PCR reaction mixtures were overlaid with 2 drops of mineral oil (Sigma) to avoid evaporation and amplification of contaminating DNA, and were performed using a 9600 thermal cycler (GeneAmp PCR system 9600, Perkin-Elmer Corporation, Norwalk, USA). In addition, water was used as a negative control in all experiments. Amplified DNA fragments were detected by electrophoresis in 1% agarose gels (NuSieve 3 : 1; FMC BioProducts, Rockland, ME, USA) in TAE buffer (40 mM Tris-base, 20 mM acetate, 2 mM EDTA). Gels were stained with ethidium bromide and photographed over a UV transilluminator (GDS 5000 UVP, San Gabriel, USA).

Restriction endonuclease analysis of N-PCR products

N-PCR products were digested with the restriction endonuclease *Hin*dII (Figure 1) according to conditions specified by the manufacturer (Boehringer Mannheim). The restricted fragments were separated by electrophoresis in 2% agarose gels in TAE buffer and visualized with UV light after ethidium bromide staining.

Sensitivity of detection

The sensitivity threshold for the detection by PCR and N-PCR of *R. solanacearum* was determined for both pure culture and plant extracts. Bacterial suspensions from 1-day-old cultures of *R. solanacearum* were adjusted to 10^8 cfu ml^{-1} and 10-fold dilutions were prepared. Three 50 µl aliquots from serially diluted bacterial suspensions were plated on modified Granada and Sequeira medium to estimate the concentration of viable *R. solanacearum* (viable cell count method). For PCR reactions, 1 ml of each dilution was boiled for 5 min, cooled on ice and 1 µl aliquot was added as template DNA to the PCR reaction mixture. The detection limit was also determined in the presence of non-infected plant extract by adding known numbers of bacterial cells (10-fold dilutions as described above) to plant extract samples.

Preparation of plant samples

Samples from wilted or healthy plants (potato and potato tubers, tomato, tobacco, eggplant, pepper and *Pelargonium asperum*) were collected. Three-cm-long pieces of stem and small fragments of potato tubers were picked, surface disinfected with ethanol, sliced with a sterile scalpel, and ground with a pestle into a sterile Petri dish containing 5 ml of buffer. Three 50 µl aliquots were plated on modified Granada and Sequeira medium to confirm the presence or absence of *R. solanacearum* in plant samples. Different buffers were assayed in order to avoid inhibition effects on PCR associated with plant tissue extracts: Tris buffer (10 mM Tris-base, pH 7.2); PP buffer (8.5 mM K_2HPO_4, 7.5 mM KH_2PO_4, pH 7.0 – 2% polyvinylpyrrolidone (PVP; Sigma); PPP buffer (PVP was replaced by polyvinylpolypyrrolidone (PVPP; Sigma); TENP and TENPP buffers (50 mM Tris, 20 mM EDTA pH 8.0, 100 mM NaCl, 2% or 5% PVP (TENP) or PVPP (TENPP). Moreover, different treatments were assayed to prevent any inhibition effect on PCR amplification, either by the addition of 5 µg or 500 ng (per PCR reaction) of bovine serum albumin (BSA) or by the dilution of plant extracts (1 : 5; 1 : 20; 1 : 100; 1 : 500) in sterile water.

Plant tissue extracts were cleared by filtration through 0.8 µm membranes (Sartorius, Göttingen, Germany) and boiled. When plant tissues were initially ground with Tris buffer, an equal volume of PP, PPP, TENP or TENPP buffer was added to the macerate before boiling.

Results

Specificity of primers

Amplification products of expected size (based on the nucleotide sequence of strain GMI1000) were observed for all tested *R. solanacearum* strains (a worldwide collection including biovars 1, 2, N2, 3, 4 and 5 strains, about 130 strains, data not shown). A fragment of 1993 bp was produced with RS30–RS31 primers for the first round PCR and two fragments (256 and 533 bp) were generated with the RS30a–RS31a, RS30b–RS31b primer sets for the second round PCR (N-PCR).

Strains belonging to 13 other bacterial species were also tested by PCR and N-PCR. No amplification occurred with DNA from these bacteria even from species closely related to *R. solanacearum*: *R. pickettii*, *R. eutropha* and *B. cepacia*. However, strains of two other closely related pathogens, *P. syzygii* and the blood disease bacterium, produced the specific PCR and N-PCR products (Figure 2).

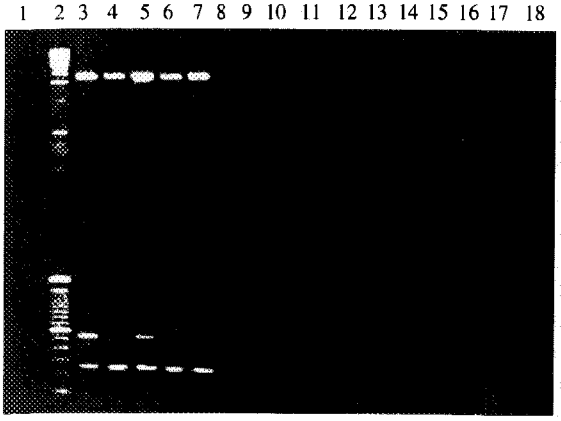

Figure 2. Gel electrophoresis of PCR products (using RS30–RS31 primers, upper part of gel) and N-PCR products (using RS30a–RS31a and RS30b–RS31b primers, lower part of gel) from *R. solanacearum* originating from Reunion Island and other bacteria. Lane 1: negative control (no template DNA); lane 2, molecular size marker GIBCO BRL (1 kb ladder on upper half of gel, 100 bp ladder on lower half of gel); lanes 3–5, *R. solanacearum* biovars 1, 2 and 3, respectively; lane 6, *Pseudomonas syzygii*; lane 7, blood disease bacterium; lane 8, *Erwinia chrysanthemi*; lane 9, *Xanthomonas* sp. pv. *mangiferae-indicae*; lane 10, *X. vesicatoria*; lane 11, *P. fluorescens*; lane 12, *P. fuscovaginae*; lane 13, *P. cichorii*; lane 14, *P. syringae* pv. *tomato*; lane 15, *Clavibacter michiganensis* subsp. *michiganensis*; lane 16, *R. pickettii*; lane 17, *Burkholderia cepacia*; lane 18, *R. eutropha*.

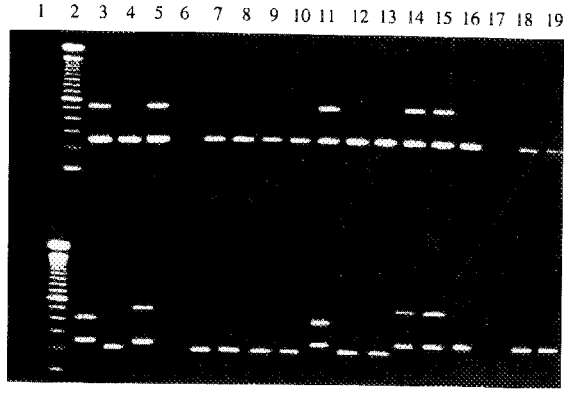

Figure 3. Gel electrophoresis of N-PCR products from cells of *R. solanacearum* and other bacteria using the two primer pairs (RS30a–RS31a and RS30b–RS31b). N-PCR products were not digested (upper part of gel) and digested (lower part of gel) with *Hin*dII. Lane 1, negative control; lane 2, 100 bp ladder GIBCO BRL; lanes 3–5, *R. solanacearum* (from Reunion Island) biovar 1 (JT525), 2 (JT509) and 3 (JT517) strains respectively; lane 6, empty; lanes 7–10, *R. solanacearum* biovar 1 (from America), JS716-JS784-JS740-JS730; lane 11, *R. solanacearum* biovar 1 (from Southern part of Africa), JS946; lane 12, biovar 2, JS895; lane 13, biovar N2, JT653; lane 14, biovar 3, JS753; lane 15, biovar 4, JS841; lane 16, biovar 5, JT661; lane 17, empty; lane 18, blood disease bacterium; lane 19, *P. syzygii*.

RFLP of N-PCR products

N-PCR products (256 and 533 bp) were digested with the restriction endonuclease *Hin*dII. Four distinct PCR-RFLP patterns were obtained for all strains. Figure 3 contains a representative set of data. For biovar 1 strains from the Southern part of Africa (Angola, Madagascar, Reunion Island, Zimbabwe), three bands were observed (115, 256 and 418 bp; one *Hin*dII restriction site within the 533 bp fragment). For biovar 1 strains originating from the Americas and two countries from Africa (Burkina Faso and Kenya), for biovar 2 and N2 strains of *R. solanacearum*, three bands were observed (34, 222 and 533 bp; one *Hin*dII restriction site within the 256 bp fragment). However, in most experiments performed with these strains, the 533 bp fragment was not visible on agarose gels. For biovar 3 and 4 strains, two bands were observed (256 and 533 bp; no *Hin*dII restriction site). Biovar 5 strains of *R. solanacearum*, the blood disease bacterium and *P. syzygii* strains gave another PCR-RFLP profile: only one amplified fragment (256 bp), with no *Hin*dII restriction site.

Detection limits in pure cultures

When pure culture of *R. solanacearum* was used, the limit of detection for bacterial cells by amplification with one round of PCR (RS30–RS31 primers) was approximately 10^6 cfu ml^{-1} (estimation by viable cell count). The sensitivity was greatly improved when N-PCR was applied since the detection level was 1000-fold greater than when the single round PCR was used. An average of 10^3 cfu ml^{-1} (1 target DNA per reaction in 1 µl of a bacterial suspension) was detected. Nevertheless, sometimes the 10^2 cfu ml^{-1} detection threshold was obtained, but it was not always reproducible (Figure 4).

When suspensions of *R. pickettii* and/or *B. cepacia* were mixed together with *R. solanacearum* suspensions, the level of detection decreased by an order of magnitude but only when the concentration of the *R. pickettii* and/or *B. cepacia* suspensions was more than 1000-fold that of the *R. solanacearum* suspension (data not shown).

N-PCR detection in plant extracts

When plant tissues were ground in Tris buffer without subsequent treatment, amplification was obtained only with a few plant samples suggesting the presence of inhibitory substances in plant extracts. Dilution of plant extracts increased the efficiency of the PCR, however, this depended upon which plant was studied (Table 3). For tomato extracts, no or a low dilution (1 : 5) was required to observe significant amplification (band of high intensity) whereas further dilutions were needed when extracts from potato (1 : 5 to 1 : 20), eggplant (1 : 5 to 1 : 20), pepper (1 : 5 to 1 : 20), tobacco (1 : 50), or *Pelargonium asperum* (1 : 500 or more) were tested. Furthermore, the PCR efficiency depended upon the age of the plant. The detection in old plants required an additional 10- or 100-fold dilution of plant extract (data not shown). Although dilution of plant extracts significantly improved the PCR efficiency, it drastically reduced the sensitivity of detection. The addition of compounds able to prevent any inhibitory effect on PCR was checked (Table 4). PP buffer was the least effective buffer since it did not improve the frequency of bacterial detection. PPP, TENP (2% PVP) or TENPP (2% PVPP) buffers increased significantly the frequency of detection in tomato, eggplant and pepper extracts. TENPP (5% PVPP) buffer or BSA (added to PCR reaction mixture) reduced most inhibition effects exhibited by all tested plants except for *Pelargonium asperum*. Only the addition of TENPP (5% PVPP) to plant extract combined with the addition of BSA to the PCR reaction mixture was able to prevent the inhibition effects exhibited by *Pelargonium asperum* extracts (Figure 5). Since no inhibition of the PCR process by the PVPP reagent was observed, PCR amplification was possible using undiluted plant tissue extracts treated with the TENPP (5% PVPP)/BSA procedure for

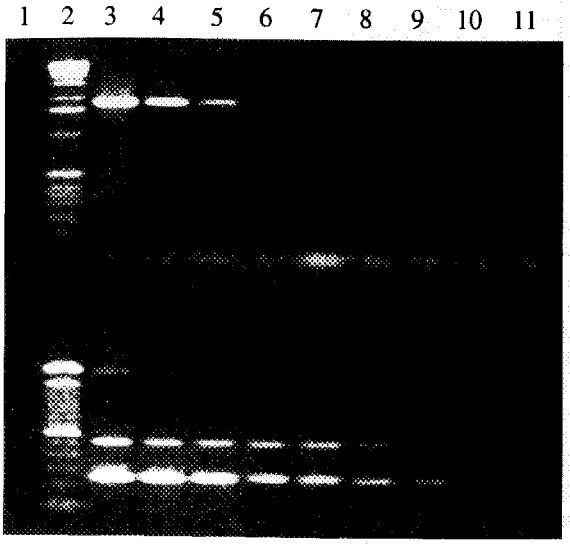

Figure 4. Gel electrophoresis of PCR (upper part of gel) and N-PCR (lower part of gel) products to assess the sensitivity of the assay for detection of *R. solanacearum* strain JT519 (biovar 3, Reunion Island) as template. Lane 1, negative control; lane 2, 1 kb ladder (upper part of gel) and 100 bp ladder (lower part of gel) GIBCO BRL; lanes 3–11, concentration (cfu ml^{-1}) estimated by viable cells count method: $\sim 10^8$, $\sim 10^7$, $\sim 10^6$, $\sim 10^5$, $\sim 10^4$, $\sim 10^3$, $\sim 10^2$, ~ 10, and 0, respectively.

Table 3. Effect of dilution of plant or tuber extracts on N-PCR detection of *R. solanacearum*

Dilution	Frequency of N-PCR detection[1,2] (number of positive samples/number of tested samples)					
	Tomato	Potato (plant and tuber)	Eggplant	Pepper	Tobacco	*Pelargonium asperum*
Undiluted	(4/5)	(1/8)	(2/5)	(2/7)	(2/5)	(0/8)
1 : 5	(5/5)	(2/6)	(5/5)	(3/5)	(2/5)	(0/5)
1 : 20	(4/4)	(4/4)	(4/4)	(4/4)	(2/3)	(0/3)
1 : 50	ND[3]	(4/4)	(4/4)	(3/3)	(4/4)	(0/6)
1 : 100	ND	(4/4)	(4/4)	(4/4)	(4/4)	(5/6)
1 : 500	ND	ND	ND	ND	ND	(6/6)

[1]Suspension of *R. solanacearum* strain JT519 (biovar 3, Reunion Island), estimated to $\sim 5 \cdot 10^6$ cfu ml^{-1} by viable cell count method, was added to each undiluted or diluted plant or tuber sample and subjected to boiling and N-PCR amplification as described in text.
[2]Detection was considered as positive if a band of expected size could be observed in ethidium bromide-stained 2% agarose gel whatever its intensity (from weak to strong).
[3]ND, not determined.

Table 4. Effect of pretreatment applied to plant extract on the frequency of detection of *R. solanacearum* by N-PCR

Treatment	cfu ml^{-1}	Frequency of N-PCR detection[1,2] (number of positive samples/number of tested samples)					
		Tomato	Potato (plant and tuber)	Eggplant	Pepper	Tobacco	*Pelargonium asperum*
No treatment	$\sim 5 \cdot 10^6$	(8/8)	(3/10)	(4/10)	(3/8)	(3/6)	(0/6)
	$\sim 5 \cdot 10^4$	(4/5)	(1/8)	(2/5)	(2/7)	(2/5)	(0/8)
BSA	$\sim 5 \cdot 10^6$	(2/2)	(2/2)	(2/2)	(2/2)	(2/2)	(0/2)
	$\sim 5 \cdot 10^4$	(4/4)	(4/4)	(4/4)	(4/4)	(4/4)	(0/6)
PP	$\sim 5 \cdot 10^6$	ND[3]	ND	ND	ND	(1/2)	(0/2)
	$\sim 5 \cdot 10^4$	(3/3)	(0/4)	(0/3)	(0/3)	(0/3)	(0/3)
PPP	$\sim 5 \cdot 10^6$	(2/2)	ND	ND	ND	ND	(0/2)
	$\sim 5 \cdot 10^4$	(3/3)	(0/4)	(3/3)	(2/3)	(1/3)	(0/3)
TENP (2% PVP)	$\sim 5 \cdot 10^6$	ND	ND	ND	ND	ND	(1/2)
	$\sim 5 \cdot 10^4$	(3/3)	(0/4)	(3/3)	(0/3)	(1/3)	(0/2)
TENPP (2% PVPP)	$\sim 5 \cdot 10^6$	ND	ND	(2/2)	(2/2)	ND	(0/2)
	$\sim 5 \cdot 10^4$	(3/3)	(2/4)	(3/3)	(2/3)	(2/3)	(0/3)
TENPP (5% PVPP)	$\sim 5 \cdot 10^6$	(2/2)	(4/4)	(2/2)	(2/2)	(2/2)	(2/3)
	$\sim 5 \cdot 10^4$	(3/3)	(4/4)	(3/3)	(3/3)	(3/3)	(0/3)
BSA+TENPP (2% PVPP)	$\sim 5 \cdot 10^6$	ND	ND	ND	(3/3)	ND	(2/3)
	$\sim 5 \cdot 10^4$	(3/3)	(4/4)	(3/3)	(3/3)	(3/3)	(2/3)
BSA+TENPP (5% PVPP)	$\sim 5 \cdot 10^6$	ND	ND	ND	ND	ND	(2/2)
	$\sim 5 \cdot 10^4$	(6/6)	(6/6)	(6/6)	(6/6)	(6/6)	(6/6)

[1]Suspensions of *R. solanacearum* strain JT519 (biovar 3, Reunion Island), estimated by viable cells count method to $\sim 5 \cdot 10^4$ and $\sim 5 \cdot 10^6$ cfu ml^{-1} respectively, were added to each plant or tuber sample and subjected to boiling and N-PCR amplification as described in text.

[2]Detection was considered as positive if a band of expected size could be observed in ethidium bromide-stained 2% agarose gel whatever its intensity (from weak to strong).

[3]ND, not determined.

the detection of *R. solanacearum* in artificially as well as in naturally contaminated plant samples. Using this procedure, the sensitivity of the N-PCR detection of *R. solanacearum* from artificially- or naturally-infected plants was not significantly different from that of pure culture.

Discussion

For the first time, sequence variation within the *hrp* gene region, assessed in previous investigation (Poussier et al., 1999), was used to develop a specific and sensitive method to detect *R. solanacearum* strains. The exploration of *hrp* genes has already proved to be useful for providing powerful tools for the detection of phytopathogenic xanthomonads (Leite et al., 1994a,b).

A specific PCR amplicon was obtained by using the selected primer pair RS30–RS31 (Poussier et al., 1999). Nevertheless, in this study, two species closely related to *R. solanacearum*, *P. syzygii* and the blood disease bacterium, gave the same RS30–RS31 size amplicon as *R. solanacearum* strains. This result was not surprising since the three species have a high degree of DNA homology (Kersters et al., 1996; Seal et al., 1993; Taghavi et al., 1996). However, these species are easily differentiated by host range, cultural and physiological properties (Eden-Green, 1994; Eden-Green and Sastraatmadja, 1990; Roberts et al., 1990). Nonetheless, the lack of specificity of the RS30–RS31 amplicon should not reduce the usefulness of the tool as a diagnostic procedure for *R. solanacearum*, since *P. syzygii* and the blood disease bacterium have been recorded only in Indonesia and on clove and banana respectively. The major interest of this PCR test was that no PCR product was obtained with other closely-related species such as *R. pickettii* and *B. cepacia* (Palleroni, 1984). Both occur commonly in soil and could be isolated from the same host as *R. solanacearum*, i.e., potato for *R. pickettii* (Seal et al., 1992b) and onion for *B. cepacia* (Girard et al., 1993; Palleroni, 1984).

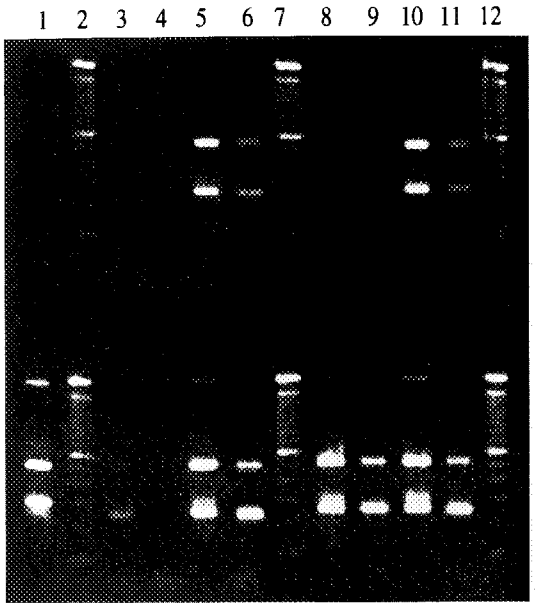

Figure 5. Gel electrophoresis of N-PCR products from cells of *R. solanacearum* (strain JT519, biovar 3, Reunion Island, as template) in plant extracts. Lane 1, negative control (upper part of gel), positive control (lower part of gel); lanes 2, 7 and 12: 100 bp ladder GIBCO BRL. *Pelargonium asperum* extract (lanes 3, 4, 8 and 9) or pepper extract were checked (lanes 5, 6, 10 and 11). Plant extracts were ground in TENPP (2% PVPP) buffer (lanes 3–6) or in TENPP (5% PVPP) buffer (lanes 8–11). BSA was added to PCR mixtures (lower part of gel) or not (upper part of gel). Concentrations of *R. solanacearum* were either $\sim 5 \cdot 10^6$ cfu ml^{-1} (lanes 3, 5, 8 and 10) or $\sim 5 \cdot 10^4$ cfu ml^{-1} (lanes 4, 6, 9 and 11).

The PCR amplification with the RS30–RS31 primer set provided a rapid and specific tool for the identification of *R. solanacearum* strains. The detection threshold (approximately 10^6 cfu ml^{-1}) was adequate to detect *R. solanacearum* in heavily infected plant material but was not sufficiently sensitive to detect early or latent infections. This low sensitivity was probably due to the fact that *hrp* gene region is present as a single copy. Indeed, when the target sequence for PCR amplification, such as rDNA operon, is present in multiple copies per genome, higher sensitivity levels can be obtained (Li and De Boer, 1995; Maes et al., 1996; Seal et al., 1993). To improve the sensitivity of a single round PCR test, two methods could be employed, either a subsequent Southern hybridization of PCR product or a second round of amplification with nested primers. The N-PCR technique was chosen because it is more rapid and less labour intensive than hybridization techniques. With N-PCR, the specificity was conserved and the sensitivity was greatly improved. The limit of detection when using either purified DNA or entire bacterial cells was lowered to 10^3 cfu ml^{-1} which corresponds to 1 cfu per reaction. Therefore, the N-PCR method should be suitable for detecting *R. solanacearum* in symptomless plants. Latent infections have been reported on potato (Buddenhagen, 1986; Ciampi et al., 1980; Nyangeri et al., 1984) and tomato (Prior et al., 1990), and may be present on many weeds (Hayward, 1991). Such infections are considered to be involved in the spread of bacterial wilt within and between countries (Hayward, 1991). Since N-PCR is a very sensitive technique, extreme care is required to avoid the risk of cross-contamination among samples and of contamination from impure PCR reagents (Hartung et al., 1996; McManus and Jones, 1995; Roberts et al., 1996).Therefore, negative control reactions were included for each test to ensure that positive results were valid. Moreover, all pipettings were done with filtered pipette tips, and pipettes that had never dispensed DNA template or PCR products were exclusively used to make-up the PCR reaction mixtures (Hartung et al., 1996). Moreover, since dead cells may be present in infected tissues and would offer target DNA, the N-PCR-RFLP procedure may lead to an overestimation of bacterial population. Furthermore, since the first step of the procedure required two rather expensive polymerases, the cost of biovar determination could be relatively high. However, compared to the classical method (Hayward, 1964), reliability and rapidity of N-PCR-RFLP offer significant advantages.

The application of the N-PCR assay to tissue extracts from potato tubers or field-grown plants failed to give consistent results due to inhibition of DNA amplification. Such inhibition, which can lead to false negative results or low detection sensitivity, has already been reported when PCR was performed to detect bacteria in plant or soil extracts (Hartung et al., 1996; Kirchhof et al., 1998; Maes et al., 1996; Minsavage et al., 1994; Picard et al., 1992). Although a wide range of inhibitors have been reported, the identity and mode of action of most of them remain unclear (Wilson, 1997). Some of these substances could inhibit the PCR reaction by denaturing or binding to the thermostable DNA polymerases (McGregor et al., 1996; Young et al., 1993), by chelating Mg^{2+} cofactor for *Taq* polymerase (Tsai and Olson, 1992), or by binding to target DNA (Steffan and Atlas, 1988). The PCR inhibitory effect of plant tissues has been attributed to polysaccharides (Demeke and Adams, 1992) or phenolic compounds such as

tannic acids (Kreader, 1996; Minsavage et al., 1994). Dilution of plant extracts was used to prevent inhibition but it caused a significant reduction in the sensitivity of the method. Therefore, dilution could not be applied to a routine detection technique. Among the various compounds considered to inactivate or remove PCR inhibitors (Audy et al., 1996; Kirchhof et al., 1998; Kreader, 1996; Leite et al., 1995; Maes et al., 1996; McGregor et al., 1996; Minsavage et al., 1994; Picard et al., 1992; Wilson, 1997), PVP, PVPP or BSA were selected. PVPP treatment appeared to be more effective than PVP. BSA added to PCR mixtures prevented inhibition except for *Pelargonium asperum* extracts. For the latter, the inhibitory effect was so strong that no amplification was observed even after treatment with PVP, PVPP or BSA. Only the combination of plant extract treatment with TENPP, including 5% PVPP, and addition of 500 ng or 5 µg of BSA to the PCR mixture successfully eliminated the inhibitory effect.

An N-PCR-RFLP procedure, based on multiplex PCR, was applied to a worldwide collection of *R. solanacearum* strains and appeared to be useful in distinguishing the two divisions of *R. solanacearum* (division I: biovars 3, 4, 5; division II: biovars 1, 2, N2) defined by Cook et al. (1989) and confirmed later (Li et al., 1993; Cook and Sequeira, 1994; Taghavi et al., 1996; Poussier et al., 1999; Seal et al., 1999). Indeed, four distinct profiles, two for each division, were observed: one for biovar 1 strains originating from the Southern part of Africa, one for American biovar 1 and biovar 2 and biovar N2 strains, one for biovars 3 and 4 strains, and one for biovar 5 strains. Although this tool is not suitable to discriminate American biovar 1 from biovar 2 or biovar N2 strains and also biovar 3 from biovar 4 strains, it could be used for accurate identification or detection of any biovar when occuring alone: for example biovar 1 in USA (Hayward, 1991) or biovar 2 in Western Europe (Stead et al., 1996) and when coexisting in the same area: for instance biovars 2, 3 and 4 in Australia, India, Indonesia, Papua New Guinea, Sri Lanka (Hayward, 1991); biovars 2, 3, 4 and 5 in China (Hayward, 1991), biovars 1, 2 and 3 in Reunion Island (Girard et al., 1993); and biovars 1 and 3 in French West Indies (Prior and Steva, 1990). If more than one biovar are present in infected plants, it could be expected that the resulting RFLP pattern would be the addition of bands relative to each biovar with or without one common band. However, when American biovar 1 strains are mixed with biovar 2 or N2 strains or when biovar 3 and biovar 4 strains are mixed, only one or two common bands could be expected.

The N-PCR-RFLP procedure applied on pretreated (with PVPP/BSA) plant samples has been developed as a powerful aid in the diagnosis of infected plants and latent infections of bacterial wilt. The application of this simple, rapid and sensitive method could be an essential step to ensure the efficiency of prophylactic measures against bacterial wilt. In addition, it could be used for monitoring the spatial distribution and colonization of *R. solanacearum* in field, soil and plants; and for epidemiological studies regarding weeds as possible carriers of *R. solanacearum* and seed as a possible vehicle for the spread of bacterial wilt.

Acknowledgements

The authors gratefully acknowledge Drs Mark Fegan, Lionel Gagnevin, Chris Hayward, Philippe Prior and Olivier Pruvost for critical comments on the manuscript; and all colleagues who have generously contributed strains to this study. This work was supported in part by a grant from the Ministère de l'Enseignement Supérieur et de la Recherche.

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