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Detection of *Ralstonia solanacearum* in Potato Tubers by Polymerase Chain Reaction

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With 3 figures

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Abstract

A new polymerase chain reaction (PCR) assay was developed for the detection of *Ralstonia solanacearum* in potato tubers. The designed primers PS-1/PS-2 based on the sequence data of the 16S rRNA gene. Using the optimized PCR protocol, it was possible to detect *R. solanacearum* cells artificially added to concentrated potato extracts in the range of 1–10 colony-forming units (CFU) per PCR reaction mixture (10–100 CFU/ml potato homogenate). No amplification products were obtained, when bacteria belonging to other species or genera were submitted to PCR under the same conditions. A total of 10 different DNA extraction methods were adapted for the isolation of *R. solanacearum* DNA from potato homogenates and were compared for their suitability as pre-PCR procedures.

Zusammenfassung

Detektion von *Ralstonia solanacearum* in Kartoffelknollen mittels Polymerase-Ketten-Reaktion

Es wurde ein neuer PCR-Test entwickelt für die Detektion von *Ralstonia solanacearum* in Kartoffel-Knollen. Die entwickelten Primer PS-1/PS-2 basierten auf Sequenzdaten des 16S rRNA Gens. Mit dem optimierten PCR Protokoll war es möglich künstlich zugegebene *R. solanacearum* Zellen in konzentrierten Kartoffel-Homogenaten zu detektieren, bei einer Nachweis-Empfindlichkeit von 1–10 CFU pro PCR-Mix (10–100 CFU pro ml Kartoffel-Homogenat). Mit dem optimierten PCR Protokoll wurden keine Amplifikationsprodukte bei Bakterien anderer Arten oder Gattungen erhalten. Außerdem wurden 10 unterschiedliche DNA-Extraktionsmethoden getestet zur Isolierung von *Ralstonia solanacearum* DNA aus Kartoffel-Homogenat und ihre Eignung für die PCR verglichen.

Introduction

Brown rot disease of potatoes is caused by the Gram-negative bacterium *Ralstonia solanacearum* (Smith) Yabuuchi et al. (1995) (syn. *Burkholderia solanacearum* (Smith) Yabuuchi et al. (1992); syn. *Pseudomonas solanacearum* (Smith) Smith). Regarded as a serious potato disease in tropical and subtropical regions of the world (Janse, 1988; Hayward, 1991), the quarantine importance of this pathogen increased in temperate Europe following several outbreaks in Sweden (Olsson, 1976), Belgium, England, France, Germany, Italy, Netherlands, Portugal and Spain (Müller, 1996; Elphinstone, 1996; Stead, 1996).

The vascular pathogen *R. solanacearum* causes wilting of potato plants and rotting of tubers; however, it also survives latently in potato tubers without causing symptoms (Ciampi et al., 1981). Transmission of the brown rot bacterium to disease-free regions may be attributed to movement of latently infected seed potatoes, where the organism remains viable and pathogenic and then causes disease under favourable conditions after planting (Janse, 1988), or to the irrigation of potatoes with contaminated surface water (Olsson, 1976; Elphinstone, 1996; Anonymous, 1997). Specific phytosanitary regulations have been introduced for the control and eradication of potato brown rot (Anonymous, 1998).

Methods currently recommended for the detection of latent *R. solanacearum* in potato (Anonymous, 1998)

include indirect immuno-fluorescent antibody staining (Janse, 1988) and culture on selective media (Elphinstone et al., 1996) with complimentary procedures including a tomato bioassay (Janse, 1988), indirect enzyme-linked immunosorbent assay (Robinson-Smith et al., 1995) and a polymerase chain reaction (PCR) assay (Seal et al., 1993). However, serological techniques can have specificity problems due to cross-reactions of polyclonal antibodies with other bacteria and limited sensitivity (Janse, 1988; Elphinstone et al., 1996). The polymerase chain reaction (PCR) offers alternatives for highly specific and sensitive identification of pathogenic bacteria. Seal et al. (1993) used *R. solanacearum*-specific oligonucleotide primers for the amplification of 16S rDNA. However, it was found that detection in potato extracts using PCR assays was not always reliable (Arulappan et al., 1996; Elphinstone et al., 1996) due to inhibition of the PCR reaction. The sensitivity of a PCR assay is limited by the sensitivity and specificity of the primers used, the recovery of the target sequences and the efficiency of the reaction. *Taq* DNA polymerase is sensitive to inhibition by factors present in biological samples (Tsai and Olsen, 1992; Abbaszadegan et al., 1993; Powell et al., 1994; Pastrik, 2000), and a number of chemicals used in the DNA extraction procedure were found to interfere with DNA amplification (Rossen et al., 1992). Therefore it is evident that any effective pre-PCR sample preparation should ensure both the removal or blocking of inhibitory substances and a high yield of amplifiable DNA.

The objective of this work was the development of a new and more reliable assay, for the extraction of DNA and amplification by PCR of specific *R. solanacearum* target sequences from crude potato tuber extracts, which would be suitable for use in routine laboratory testing programmes.

Materials and Methods

Bacterial strains and culture conditions

Bacteria (Table 1) were obtained either from the Göttinger Collection of Phytopathogenic Bacteria (GSPB; Göttingen, Germany), or from the German Collection of Micro-organisms and Cell Cultures (DSMZ; Braunschweig, Germany), National Collection of Plant Pathogenic Bacteria (NCPPB; Central Science Laboratory, York, UK), or from IACR Rothamsted (R; Harpenden, Hertfordshire, UK). *Ralstonia 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 YPN agar (Rhodes, 1959) at ambient temperature.

Sample preparation

In accordance with the official EC method for detection and diagnosis of *R. solanacearum* in potatoes (Anonymous, 1998), composite samples each consisting of

Table 1
Bacterial strains used in this study

Strains	Source and culture code
Ralstonia solanacearum	DSM 9544
	GSPB 1958
	GSPB 1960
	GSPB 2124
	GSPB 2126
	R 842 = NCPPB 325
	R 301 = NCPPB 4027
	R 309 = NCPPB 3980
	NCPPB 1331
	R 578 = NCPPB 3985
	R 583 = NCPPB 3986
	R 278 = NCPPB 3992
	R 284 = NCPPB 3993
	R 277 = NCPPB 4001
	R 279 = NCPPB 4002
	R 288 = NCPPB 4011
	R 292 = NCPPB 4012
Ralstonia eutrophus	DSM 531
	NCPPB 4048
	NCPPB 4049
Ralstonia pickettii	DSM 6297
	NCPPB 4075
	NCPPB 4076
Burkholderia andropogonis	DSM 9511
	NCPPB 1127
	NCPPB 2869
Burkholderia caryophylli	DSM 50341
	NCPPB 353
	NCPPB 2151
Burkholderia cepacia	DSM 7288
	NCPPB 4074
	NCPPB 945
	NCPPB 946
Burkholderia gladioli	DSM 4285
Burkholderia glumae	DSM 9512
	NCPPB 3708
Burkholderia vandii	DSM 9510
Burkholderia plantarii	NCPPB 3590
Clavibacter michiganensis ssp. *sepedonicus*	DSM 46300
	GSPB 1522
	GSPB 2238
	GSPB 2249
Clavibacter michiganensis ssp. *michiganensis*	DSM 20134
	DSM 46364
	GSPB 382
	GSPB 390
Clavibacter michiganensis ssp. *insidiosus*	DSM 20157
	GSPB 2225
	GSPB 29
Clavibacter michiganensis ssp. *tesselarius*	DSM 20741
Clavibacter michiganensis ssp. *nebraskensis*	DSM 20400
	DSM 20401
	DSM 7483
Erwinia carotovora ssp. *atroseptica*	DSM 60424
	GSPB 401
Erwinia carotovora ssp. *carotovora*	DSM 30168
	GSPB 133
Erwinia chrysanthemi	GSPB 421
	DSM 30177
Erwinia rhapontici	GSPB 454
	GSPB 455
Pseudomonas syringae pv. *atrofaciens*	GSPB 1440
	GSPB1392
Pseudomonas syringae pv. *morsprunorm*	DSM 50302
Pseudomonas syringae pv. *phaseolicola*	GSPB 1495
	GSPB 567

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 water.

Potato samples

Composite samples of 200 seed potato tubers were submitted to Pflanzenschutzamt Hannover as required for official ring-rot surveys of seed potatoes and were tested for *R. solanacearum* using a PCR assay with primers OLI I and Y2 (Seal et al., 1993) and the designed primers PS-1/PS-2. Approximately 500 randomly selected composite samples of 60 different cultivars were tested. Sample preparation was performed by the procedure described above.

Sensitivity of PCR

To determine the detection limit of the PCR, a culture of *R. solanacearum* [GSPB 1958; approximately 10^8 colony-forming units (CFU)/ml] was serially diluted by 10-fold increments in sterile water. Aliquots (100 μl) of the serial dilutions were transferred into plastic bags containing tissue samples (200 tissue cores) of healthy potatoes. The tissue cores of the healthy potatoes were removed from a homogeneous potato tuber mixture of the potato cultivars Agria, Bonanza, Cilena, Granola and Linda. Samples were macerated using the 'Homex' apparatus and treated as described above. The resulting artificially inoculated potato homogenates (1 ml, respectively) were used for the DNA extraction series. The experiment was repeated at least three times. Concentrations of viable bacteria in suspension used as inoculum were estimated as the number of CFU which developed after plating 100 μl of the serial dilutions on casamino peptone glucose agar (Kelman, 1954).

DNA extraction

Method 1: Cell lysis by heating to 96°C (Seal et al., 1993)

Potato macerate (100 μl) was placed in heating block for 4 min at 96°C, cooled for 10 min on ice and centrifuged for 3 min at 13 000 × *g* at 4°C. The supernatant was serially diluted 1 : 10 and 1 : 100 in sterile water. Aliquots of 2 μl were added to the PCR reaction mix.

Method 2: Easy-DNA-Extraction Kit

DNA was extracted as described by Pastrik, 2000. Potato macerate (100 μl) was mixed with 220 μl of 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 (Boehringer Mannheim, Germany) stock solution (50 mg/ml 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, The Netherlands). Solution A (220 μl) was added to the homogenate, 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, DNA was precipitated with 96% ethanol and the resulting pellet was washed with 80% ethanol. After the final centrifugation, the DNA was dried and the pellet was dissolved in 100 μl of sterile water. 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* at 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) and further DNA purification was performed by the procedure described above.

Method 3: Sodium dodecyl sulphate treatment (modified from Dellaporta et al., 1983)

Potato macerate (100 μl) was mixed with 220 μl of 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 (Boehringer Mannheim) stock solution (50 mg/ml in 10 mM Tris-HCl, pH 8.0) was added, and the sample was incubated for 30 min at 37°C. Then 400 μl of extraction buffer (500 mM NaCl; 100 mM Tris-HCl, pH 8.0; 50 mM EDTA, pH 8.0; 10 mM mercaptoethanol) were added and the homogenate was mixed. After addition of 40 μl of 20% sodium dodecyl sulphate (SDS) and mixing, the homogenate was incubated for 10 min at 65°C. Then 250 μl of 5 M potassium acetate was mixed in, the sample was incubated for 20 min on ice and centrifuged at 25 000 × *g* for 20 min. The pellet was discarded and the supernatant transferred to a fresh tube. Nucleic acids were precipitated twice, once with 0.6 vol. of isopropanol and once with 0.1 vol. of 3 M sodium acetate : 2.5 vol. of ethanol. After the final centrifugation the pellet was washed with 70% ethanol and dissolved in 100 μl sterile water. The resulting DNA extract was serially diluted 1 : 10 and 1 : 100 in sterile water. Aliquots of 5 μl were added to the PCR reaction mix.

Method 4: CTAB (2%) treatment (modified from Tinker et al., 1993)

Potato macerate (100 μl) was mixed with 220 μl of 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 (Boehringer Mannheim) stock solution (50 mg/ml

in 10 mM Tris-HCl, pH 8.0) was added, and the sample was incubated for 30 min at 37°C. Then 500 µl CTAB buffer (1.4 M NaCl; 100 mM Tris-HCl, pH 8.0; 2% (w/v) cetyl trimethyl ammonium bromide (CTAB); 20 mM EDTA, pH 8.0) was added and the homogenate was mixed. The sample was incubated for 30 min at 65°C and centrifuged at 25 000 × *g* for 15 min. The pellet was discarded and the supernatant transferred to a fresh tube and then extracted with 500 µl of chloroform. After centrifugation for 5 min at 25 000 × *g*, the aqueous phase was transferred into a new tube and nucleic acids were precipitated with 0.6 vol. of isopropanol, washed with 80% ethanol and the pellet was resuspended in 100 µl sterile water. The resulting DNA extract was serially diluted 1 : 10 and 1 : 100 in sterile water. Aliquots of 5 µl were added to the PCR reaction mix.

Method 5: Plant DNAZOL reagent (Life Technologies, Germany)

Potato macerate (100 µl) were processed according to the instructions of the manufacturer for plant tissue. The resulting DNA extract was serially diluted 1 : 10 and 1 : 100 in sterile water. Aliquots of 5 µl were added to the PCR reaction mix.

Method 6: Genome Clean Kit (AGS, Germany)

Potato macerate (100 µl) were processed according to the instructions of the manufacturer for genomic DNA using 200 µl buffer 1. Precipitation of DNA was performed by adding 450 µl sterile water and 50 µl buffer 2 to the aqueous phase. The resulting DNA extract was serially diluted 1 : 10 and 1 : 100 in sterile water. Aliquots of 5 µl were added to the PCR reaction mix.

Method 7: Nucleon PhytoPure Kit for plant tissue (Amersham, Germany)

Potato macerate (100 µl) were processed according to the instructions of the manufacturer for small samples using 300 µl of reagent 1, 100 µl of reagent 2 and 100 µl of Nucleon-Resin. The resulting DNA extract was serially diluted 1 : 10 and 1 : 100 in sterile water. Aliquots of 5 µl were added to the PCR reaction mix.

Method 8: InstaGene Purification Matrix (Biorad, Germany)

Potato macerate (100 µl) were processed according to the instructions of the manufacturer for bacteria using 200 µl InstaGene matrix. The resulting DNA extract was serially diluted 1 : 10 and 1 : 100 in sterile water. Aliquots of 5 µl were added to the PCR reaction mix.

Method 9: NucleoSpin Plant Kit (Macherey-Nagel, Germany)

Potato macerate (100 µl) were processed according to the instructions of the manufacturer for plant tissue using 250 µl buffer C1 and 300 µl buffer C4. The resulting DNA extract was serially diluted 1 : 10 and 1 : 100 in sterile water. Aliquots of 5 µl were added to the PCR reaction mix.

Table 2
Primer sequences and sizes of amplified DNA-fragments

Primer	Primer sequence 5′-3′	Size of PCR product
PS-1	agt cga acg gca gcg ggg g	553 bp
PS-2	ggg gat ttc aca tcg gtc ttg ca	
Y-2	ccc act gct gcc tcc cgt agg agt	288 bp
OLI-1	ggg ggt agc ttg cta cct gcc	

Method 10: Dneasy Plant Kit (Quiagen, Germany)

Potato macerate (100 µl) were processed according to the instructions of the manufacturer for plant tissue using 300 µl buffer AP-1, 4 µl RNase A solution, 130 µl buffer AP-2, a QIAshredder spin column and 100 µl buffer AE. The resulting DNA extract was serially diluted 1 : 10 and 1 : 100 in sterile water. Aliquots of 5 µl were added to the PCR reaction mix.

Primers

Primers employed in this study (Table 2) were high-performance liquid chromatography-purified and purchased from Life Technologies (Germany). For the primer design of the primers PS-1/PS-2 the 16S rRNA partial gene sequences obtained from the GenBank for *Ralstonia* and *Burkholderia* species (accession numbers AH004174, M32021, U96933, X67035-X67041, X80287) were aligned using the computer package CLUSTALW (Thompson et al., 1994). On the basis of hypervariable regions within the 16S rRNA sequences, forward (PS-1) and reverse (PS-2) primers were designed with a predicted PCR product of 553 bp. Amplification with primer OLI I and Y2(Seal et al., 1993) generated a PCR product of 288 bp.

PCR amplification

PCR was performed in a PTC 200 thermocycler (MJ Research, USA). For the amplification with primers PS-1/PS-2 the PCR reaction mixture (25 µl) 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); 0.1% bovine serum albumin fraction V (Serva, Germany); 0.2 µM each of primers PS-1 and PS-2; 0.5 U *Taq* DNA polymerase (Life Technologies) and 2–5 µl of the DNA solution. 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, 68°C for 30 s and 72°C for 45 s. After the final reaction cycle, the mixture was kept at 72°C for 5 min and stored at 4°C. Additional amplifications were performed using primer OLI I and Y2 described by Seal et al. (1993). After the PCR, 12 µl aliquots of the reaction mixture were resolved by electrophoresis on a 2% agarose gel, and DNA fragments were visualised by staining in 0.5 µg/ml ethidium bromide.

Restriction analysis

The specificity of the PCR product amplified with primer PS1/PS2 was confirmed by restriction analysis with *Taq*I. Samples of 10 µl of PCR product were digested with 5 U of *Taq*I (Boehringer Mannheim) 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 ethidium bromide.

Results

To test the specificity of the designed primers PS-1/PS-2, amplification was carried out with genomic DNA of all bacterial strains listed in Table 1 and extracted with Easy-DNA extraction kit (Invitrogen). The primers PS-1/PS-2 (Table 2) amplified a specific DNA fragment in the size of 553 bp with DNA of all *Ralstonia solanacearum* strains (Fig. 1, lane 1). The specificity of the amplified PCR products was confirmed by restriction analysis with *Taq*I, whose restriction site is present in the *R. solanacearum*-specific PCR product. The restriction fragments obtained from the *R. solanacearum*-specific DNA fragment were 457 bp and 96 bp in size (Fig. 1, lane 2). Primers PS-1/PS-2 generated from *Ralstonia pickettii* strains a PCR product approximately 60 bp smaller, which was easily discernible after agarose gel electrophoresis and had different digestion sites in restriction analysis with *Taq*I. Amplification products were not obtained from DNA of bacteria of other species or genera (data not shown).

Table 3 shows the results obtained with a total of 10 methods adapted for extraction of *R. solanacearum* DNA from potato homogenates and compared for their suitability as pre-PCR procedures. The methods tested were based on either (i) crude cell lysis by heating without purification steps (method 1); (ii) conventional procedures with chloroform or phenol/chloroform extraction and subsequent precipitation of the DNA by ethanol or isopropanol (methods 2–7); (iii) DNA-binding resins (method 8); or (iv) spin column purification (methods 9 and 10).

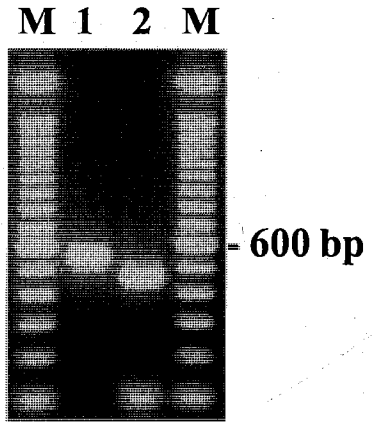

Fig. 1 Restriction analysis with *Taq*I (Boehringer Mannheim) of *Ralstonia solanacearum*-specific PCR products amplified with primers PS-1/PS-2. Lane 1 and 2, undigested and digested specific PCR product from genomic DNA of *R. solanacearum* (GSPB 1958); lanes M, DNA size marker (100 bp ladder, Life Technologies)

All of these methods were tested in at least three DNA extraction series. The sensitivity was defined in terms of the minimum number or range of cells detected per ml of potato homogenate following successful PCR amplification with primers Ps-1/Ps-2 in the three repeated DNA extraction series. The reliability of each DNA extraction method was defined in terms of consistency of the results in the three repeated DNA extraction series with respect to the number of failed target DNA amplifications from samples containing bacterial concentrations higher than the sensitivity

Table 3
Results of the different DNA extraction methods tested

DNA extraction method	Sensitivity [a]	Dilution of DNA extract prior to PCR	DNA extraction failures [b]	Reliability [c]
Method 1: Cell lysis by heating	10^3	Yes	4	Low
Method 2: Easy-DNA	10^1–10^2	No	0	High
Method 3: SDS	10^3	Yes	0	High
Method 4: CTAB	10^3	Yes	0	High
Method 5: Plant DNAzol	10^4–10^5	Yes	5	Low
Method 6: Genome Clean	10^3	Yes	2	Medium
Method 7: Nucleon-PhytoPure	10^3–10^4	Yes	2	Medium
Method 8: Insta Gene	10^5–10^6	Yes	3	Low
Method 9: NucleoSpin Plant	10^3	No	2	Medium
Method 10: Dneasy Plant	10^3	No	2	Medium

[a] Minimum numbers or range of cells detected per ml of potato homogenate indicated as successful PCR amplification with primers Ps-1/Ps-2 in 3 repeated DNA extraction series.
[b] Observed DNA extraction failures from samples containing bacterial concentrations higher than the sensitivity threshold indicated as unsuccessful PCR amplification in three repeated DNA extraction series.
[c] Observed DNA extraction failures in potato homogenate samples containing bacterial concentrations higher than the sensitivity threshold indicated as unsuccessful PCR amplification in three repeated DNA extraction series, no failure = high, 1–2 failures = medium, > 2 failures = low.

threshold (high = no failure, medium = 1–2 failures, low = > 2 failures).

Most of the methods tested required a 1 : 10-dilution step of the DNA extract prior to PCR (methods 1, 3–8) to circumvent inhibitory effects resulting in reduced sensitivity of detection. With methods 1, 5 and 8 more than two amplification failures were observed resulting in low reliability. Filtration steps during spin column purification (methods 9, 10) impaired DNA recovery due to impermeability problems of the potato homogenates and the potential sensitivity and reliability was not realized. The initial experiments indicated the highest sensitivity and reliability with DNA extracts purified using the Easy-DNA extraction kit (method 2). On the basis of these results further optimization experiments were performed only with the Easy-DNA extraction kit. Additionally, α-amylase was tested in the DNA extraction procedure to eliminate possible inhibitory effects by starch within potato tuber homogenates, however, no improvement of the sensitivity was observed (data not shown).

In further PCR optimization experiments the influence of additives or cosolvents in the PCR reaction was tested. In the present experiments only the addition of 0.1% bovine serum albumin (BSA) to the PCR mix influenced the efficiency of the PCR and resulted in an enhanced yield of the amplification products. Additionally, the use of BSA enabled amplification of some but not all undiluted DNA extracts isolated by SDS (method 3) and CTAB (method 4) procedures. Other additives, including formamide, glycerol, Tween 20 and dimethyl sulphoxide (DMSO), were also tested but did not increase sensitivity of the PCR (data not shown).

Using the optimized PCR protocol with primers PS-1/PS-2 and Easy-DNA extraction method (method 2), it was possible to detect *R. solanacearum* cells artificially added to concentrated potato extracts in the range of 1–10 CFU per PCR reaction mixture (10–100 CFU per ml potato homogenate) (Fig. 2).

Approximately 500 randomly selected composite potato tuber samples were tested by PCR in surveys for *R. solanacearum*. Samples were extracted with Easy-DNA-kit (method 2) and amplified with primers OLI I/Y2 (Seal et al., 1993) and primers PS-1/PS-2. However, when using primers OLI I/Y2, weak DNA fragments were amplified from 45 out of 500 healthy potato tuber samples with similar but not equal size to that of the expected specific PCR product of 288 bp (Fig. 3a, lane 1–4). These DNA fragments appeared independent of the tested potato cultivar and probably due to nonspecific 'background' amplification of unknown origin. No background amplification was generated from the same potato tuber samples with primers PS-1/PS-2 (Fig. 3b, lane 1–4).

Discussion

Theoretically, the PCR technique is able to detect as few as one single copy of target DNA. The sensitivity of this method is limited by the recovery of the target sequences and the efficiency of the PCR reaction. *Taq* polymerase is sensitive to inhibition by compounds present in biological samples (Shioda and Marakami-Muofushi, 1987; Powell et al., 1994; Rogers et al., 1996; Pastrik, 2000) and a number of chemicals used in DNA extraction procedures were found to interfere with DNA amplification (Rossen et al., 1992). Depend-

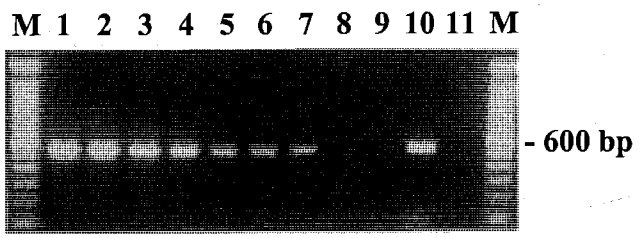

Fig. 2 Sensitivity of a PCR assay for detection of *Ralstonia solanacearum* in potato homogenate using primers PS-1/PS-2. Healthy potato tuber extracts were mixed with 10-fold dilutions of *R. solanacearum* cells. Lanes 1–8, dilutions of *R. solanacearum* cells ranging from 1×10^6 to 0.1 CFU per PCR reaction mixture; lane 9, healthy potato sample; lane 10, genomic DNA of *R. solanacearum* (GSPB 1958); lane 11, negative control; lanes M, DNA size marker (100 bp ladder, Life Technologies)

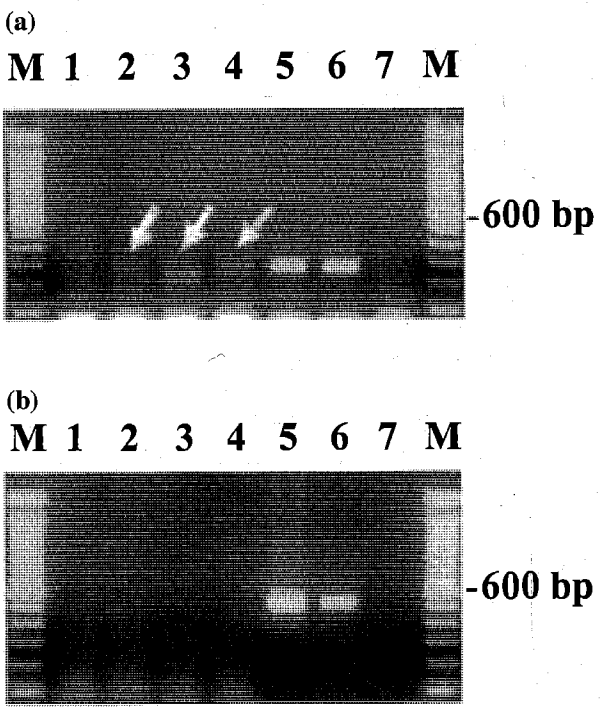

Fig. 3 Representative PCR results in surveys for *Ralstonia solanacearum* in potato seed samples extracted with Easy-DNA Kit and amplified with (a) primers OLI I/Y2; and (b) primers PS-1/PS-2. Lanes 1–4, DNA of various healthy potato seed samples; lane 5, DNA of potato extracts mixed with *R. solanacearum* cells (2×10^5); lane 6, genomic DNA of *R. solanacearum* (GSPB 1958); lane 7, negative control; lanes M, DNA size marker (100 bp ladder, Life Technologies, Germany). Arrows indicate 'background' amplification

ing on the sample type, specific requirements are placed on the method of DNA isolation in respect to purity and yield of the DNA obtainable. Several methods have been described (Lee and Taylor, 1990; Keil and Griffin, 1994; Rogers et al., 1996; Wilson et al., 1996) and numerous commercial kits are available for DNA isolation from plant tissues, based on different methodologies. Most of these methods had been optimized for DNA isolation from leaf tissue, but in this study they were adapted and compared for the detection of *R. solanacearum* in potato tuber homogenates, known to be a particularly difficult medium (Rogers et al., 1996). In the present experiments, in most cases it was not possible to reproduce the described sensitivity and reliability of the DNA extraction methods tested. This may be due to insufficient optimization of the methods or detrimental effects of the adaptations used. A disadvantage of methods based on conventional procedures are the use of hazardous chemicals such as phenol or chloroform. Methods using spin column purification replace these chemicals by guanidine-salts, however, these are also hazardous chemicals. Whichever procedure is employed, care must be taken to eliminate substances known to inhibit PCR or at least to keep them at ineffective concentrations. This can be achieved by dilution of the DNA extracts but with simultaneous dilution of the target sequence and therefore reduced sensitivity of the pathogen detection.

In this study, the most reliable results were obtained using the optimized Easy-DNA extraction kit and primers PS-1/PS-2 for the detection of *R. solanacearum* in potato tuber homogenates. The detection level was consistently in the range of 1–10 CFU per PCR reaction mixture, which was similar to that found by Seal et al. (1993). Additionally, the DNA extraction method described here is amenable for the Gram-positive bacterium *Clavibacter michiganensis* ssp. *sepedonicus* (Pastrik, 2000), the causal agent of the economically important bacterial ring rot disease of potato (Anonymous, 1993), thus enabling dual testing of the same extracts for both important quarantine organisms.

Co-solvents or additives were reported to be useful for some PCR reactions (Winship, 1989; Smith et al., 1990; Bereswill et al., 1995). The mechanism underlying the effect of these chemicals on PCR are largely unknown (Rolfs et al., 1992). In the present study only the addition of bovine serum albumin (BSA) to the PCR mix influenced the efficiency of the PCR reaction.

Seal et al. (1993) developed a PCR assay for the detection of *R. solanacearum* in potato tuber homogenates. In this assay, DNA was isolated by crude cell lysis without purification steps. However, in the present experiments this extraction method was impaired by inconsistent reliability and reduced sensitivity. Furthermore, the primers OLI I/Y2 amplified from some healthy potato tuber samples a nonspecific product of similar size to that expected from *R. solanacearum*, resulting in difficult interpretation of these results. Using the newly developed primers PS-1/PS-2 no 'background' amplification was observed with the same healthy potato tuber samples.

Some prepurification methods such as enrichment procedures (Schaad et al., 1995; Elphinstone et al., 1996; Toth et al., 1999) have been developed to purify bacterial cells prior to PCR. In this study, pre-enrichment steps were not examined because one of the objectives was rapid detection and pre-enrichment can be either time consuming or labour-intensive.

In conclusion, a reliable and sensitive PCR assay has been developed for the detection of *R. solanacearum* in potato tuber homogenates. Further investigations have been initiated to determine its applicability as a screening assay for the detection of *R. solanacearum* in potato tuber homogenates.

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