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Detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato tubers by multiplex PCR with coamplification of host DNA

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Abstract

A new multiplex PCR assay was developed for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* in potato tubers. The assay combines two different tests in one reaction mixture. First, a highly specific and sensitive detection of the pathogen and second, an indicator test for successful amplification (internal PCR control), which monitors potentially false-negative PCR results, caused by inhibition of the PCR. For the simultaneous amplification of two different targets in one reaction mixture, a mix of two different primer sets was used. For the detection of *C. michiganensis* subsp. *sepedonicus*, a pathogen-specific primer set PSA-1/PSA-R was used, based on the intergenic spacer region of the 16S–23S rRNA genes of *C. michiganensis* subsp. *sepedonicus*. For the simultaneous amplification of the internal PCR control, the plant-specific primer set NS-7-F/NS-8-R was employed, permitting amplification of target sequence from plant DNA present in DNA extractions from potato core fluid. The applicability of the multiplex PCR was verified in 3500 composite samples of 200 seed potato tubers from 143 different cultivars in a survey for *C. michiganensis* subsp. *sepedonicus* by parallel testing using immunofluorescence, a bioassay in eggplant seedlings and multiplex PCR.

Introduction

The gram-positive coryneform bacterium *Clavibacter michiganensis* subsp. *sepedonicus* is the causal agent of bacterial ring rot disease of potato, an economically important disease for the potato seed industry. This pathogen causes wilting of potato plants and degradation of vascular tissue in rotting tubers. However, it can survive latently in potato tubers for long periods without causing symptoms (Zielke and Naumann, 1984). The main routes of transmission of the bacterium are via infected seed potatoes or contaminated potato handling equipment (Mansfield-Giese, 1997; Nelson, 1980). For the control and eradication of potato ring rot, existing phytosanitary regulations (Anonymous, 1977) rely on the availability of disease-free seed tubers. Current test procedures include serological techniques such as ELISA and immunofluorescence (IF)-assay (Anonymous, 1993; DeBoer et al., 1988; Franken et al., 1993). However, they are not always reliable because of cross-reactions with other bacteria (Calzolari et al., 1982; Bishop et al., 1988; DeBoer, 1982) and limited sensitivity (DeBoer et al., 1996; 1994).

Several PCR assays have been developed for specific and sensitive detection of *C. michiganensis* subsp. *sepedonicus* (Lee et al., 1997; Li and DeBoer, 1995; Mills et al., 1997; Pastrik and Rainey, 1999; Schneider et al., 1993). In these assays, only pathogen-specific primer sets are employed ('uniplex PCR'). However, the interpretation of negative results is difficult because these may be caused by the absence of the pathogen or failed DNA isolation or by inhibition of the PCR reaction. In the two latter cases false-negative

results are obtained. Taq DNA polymerase is sensitive to inhibition by factors present in biological samples (Powell et al., 1994; Tsai and Olsen, 1992), and a number of chemicals used in the DNA extraction procedure were found to interfere with DNA amplification (Rossen et al., 1992). The exclusion of false-negative results is essential for the use of PCR-based tests as screening assays.

Hu et al. (1995) developed a competitive PCR, in which an internal standard DNA template was artificially added to the 'uniplex' PCR mix. This standard DNA served as an internal PCR control and potentially detected inhibitors in the DNA extracts, but did not indicate the success of DNA isolation. This PCR assay employed specific primers (Schneider et al., 1993) derived from the indigenous plasmid pCS1 of *C. michiganensis* subsp. *sepedonicus* (Mogen and Oleson, 1987). However, this plasmid is not present in all *C. michiganensis* subsp. *sepedonicus* strains (Mogen et al., 1988; Schneider et al., 1993) and assays based on this plasmid have been shown to cross-react with *C. michiganensis* subsp. *insidiosus* (Drennan et al., 1993).

Multiplex PCR amplification (Chamberlain et al., 1988) provides a means of reliable pathogen detection in routine testing. Since several primer sets are used in the same PCR assay, simultaneous amplification of more than one DNA region of interest is possible in a single reaction mixture. Multiplex PCR is already widely applied in detection of *Escherichia coli*, *Salmonella* spp. and other bacterial pathogens (Bej et al., 1990; 1991; Rodriguez et al., 1991; Way et al., 1993).

This study describes the development of a multiplex PCR for the detection of *C. michiganensis* subsp. *sepedonicus* in potato tubers, in which coamplification of host DNA provides a novel internal PCR control thus improving the reliability of the PCR assay for routine screening of potatoes for phytopathogenic bacteria.

Materials and methods

Bacterial strains and growth conditions

Bacteria tested in this study (Table 1) were obtained either from the Göttingen Collection of Phytopathogenic Bacteria (GSPB, Göttingen, Germany) or the German Collection of Microorganisms and Cell Cultures (DSMZ, Braunschweig, Germany). All *Clavibacter* spp. were grown on yeast extract glucose mineral salts agar (YGM-Agar) (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 *C. michiganensis* subsp. *sepedonicus* in potatoes (Anonymous, 1993) composite samples consisting of 200 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

Table 1. Bacteria used in this study

Strains	No. of strains	Sources and strain designation
Clavibacter michiganensis subsp. *sepedonicus*	7	GSPB 1522, 2237, 2238, 2242, 2249, 2250, DSM 46300
C. michiganensis. subsp. *michiganensis*	6	GSPB 382, 390, 392, 2315, DSM 20134, 46364
C. michiganensis subsp. *insidiosus*	3	GSPB 29, 2225, DSM 20157
C. michiganensis subsp. *tesselarius*	1	DSM 20741
C. michiganensis subsp. *nebraskensis*	4	DSM 7483, 20400, 20401, GSPB 2223
Erwinia carotovora subsp. *carotovora*	7	GSPB 133, 425, 440, 445, 1260, 1315, DSM 30168
E. carotovora subsp. *atroseptica*	8	GSPB 401, 408, 410, 1255, 1401, 1698, 2232, DSM 60424
E. chrysanthemi	3	GSPB 413, 415, 421
Pseudomonas syringae pv. *phaseolicola*	4	GSPB 567, 1495, 2203, 2204
P. syringae pv. *syringae*	5	GSPB 870, 1005, 1150, 1441, 1686
P. fluorescens	3	GSPB 1499, 1560, 1713
Ralstonia solanacearum	6	GSPB 1958, 1960, 2113, 2124, 2126, DSM 9544

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 pellet was resuspended in 1 ml sterile water. Samples of individual leaves or small stem pieces (3–4 cm in length) from tomato (cv. Moneymaker) or eggplant (cv. Black Beauty) were transferred into plastic bags and treated as described above.

Sensitivity of PCR

To determine the detection limit of the PCR, a culture of *C. michiganensis* subsp. *sepedonicus* (GSPB 1522; 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 with the 'Homex' apparatus and treated as described above. Concentrations of viable bacteria were estimated as the number of CFU which developed after plating 100 μl of the serial dilutions on YGM medium.

DNA extraction

DNA was extracted as described by Pastrik and Rainey (1999). 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, 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.

Primers

Primers employed in this study (Table 2) were HPLC-purified and purchased from Roth (Karlsruhe, Germany). For the specific amplification of *C. michiganensis* subsp. *sepedonicus*, the pathogen-specific primer set PSA-1/PSA-R was used, which is based on the intergenic spacer region (16S–23S rRNA) of *C. michiganensis* subsp. *sepedonicus* (Pastrik and Rainey, 1999) with a predicted PCR product of 502 bp. For the amplification of the internal PCR control, the primer set NS-7-F/NS-8-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 discoideum* and *Stylonicha pustulata* (Dams et al., 1988) and they amplify a DNA fragment of 377 bp from potato, eggplant and tomato.

PCR amplification

PCR was performed in a PTC 200 thermocycler (MJ Research, USA). 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, Germany); 0.1% bovine serum albumin fraction V (Serva, Germany); 0.2 μM each of primers PSA-1/PSA-R and 0.04 μM each of primers NS-7-F/NS-8-R; 1 U 'Platinum' Taq DNA polymerase (Life Technologies, Germany) and

Table 2. Primer sequences and sizes of amplified DNA fragments

Primer	Primer sequence 5′–3′	Size of PCR product
PSA-1	ctc ctt gtg ggg tgg gaa aa	502 bp
PSA-R	tac tga gat gtt tca ctt ccc c	
NS-7-F	gag gca ata aca ggt ctg tga tgc	377 bp
NS-8-R	tcc gca ggt tca cct acg ga	

5 µl of the DNA solution. The following PCR conditions were used: initial denaturation at 95 °C for 3 min, followed by 10 reaction cycles of 95 °C for 1 min, 64 °C for 1 min and 72 °C for 1 min, and 25 reaction cycles of 95 °C for 30 s, 62 °C for 30 s and 72 °C for 1 min. The rate of heating (ramp) from 64 (or 62 °C) to 72 °C was regulated to 0.5 °C/s. After the final reaction cycle, the mixture was kept 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 on a 2% agarose gel, and DNA fragments were visualized by staining in 0.5 µg/ml ethidium bromide.

Restriction analysis

The specificity of the PCR was confirmed by restriction analysis with *Bgl*II. Samples of 10 µl of PCR product were digested with 5 U of *Bgl*II (Boehringer Mannheim, Germany) at 37 °C for 2 h. Restriction products were analysed by electrophoresis on a 2% agarose gel and visualized by staining with 0.5 µg/ml ethidium bromide.

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 in parallel using the IF-assay (Anonymous, 1993) and multiplex PCR. Approximately 3500 composite samples of 143 different cultivars (Table 3) were tested. Sample preparation was performed by the procedure described above.

Immunofluorescence (IF)-test

The IF-test was performed according to the standard EC-protocol for the detection and diagnosis of the ring rot bacterium in batches of potato tubers (Anonymous, 1993). For screening, a polyclonal anti-*C. michiganensis* subsp. *sepedonicus* antiserum (obtained from Dr. Rohloff, Federal Biological Research Centre for Agriculture and Forestry (BBA), Braunschweig, Germany) was used. Bacterial concentrations were estimated from visual counts of typical fluorescing cells observed in 60 microscope fields and recorded as the mean number of immunofluorescing units (IFU) per microscope field from which the number of cells (N) per ml of resuspended pellet were calculated. Samples with > 3 IFU per microscope field in undiluted resuspended pellet (IFU per microscope field $\times$ dilution factor) were considered positive for infection (threshold > 3 IFU per microscope field $= 1 \times 10^5$ cells per ml of undiluted resuspended pellet) according to the standard EC-protocol (Anonymous, 1993). Samples which were IF- and/or PCR-positive were additionally tested using a monoclonal antibody (9A1, Agdia Inc., USA) as described by DeBoer and McNaughton (1986).

Table 3. Potato cultivars tested in this study

Adretta	Elvira	Lady Claire	Rita
Afra	Exempla	Lady Rosetta	Rosara
Agave	Exquisa	Leyla	Rosella
Agria		Likaria	Roxy
Aiko	Fasan	Linda	Rustica
Albatros	Fausta	Liu	Russet Burbank
Ardenta	Filea	Lyra	
Arkula	Florijn		Sandra
Arnika	Franka	Marabel	Sanira
Artis	Friesländer	Mentor	Satina
Assia		Milva	Saturna
Astarte	Gambria	Miriam	Sava
Atica	Gloria	Molli	Secura
Aula	Goya		Selma
Aurora	Grandifolia	Natalie	Sempra
	Granola	Nicola	Serafina
Belita	Grata	Nagore	Sibu
Berber			Sieglinde
Berolina	Hansa	Padea	Simone
Bintje	Hela	Paola	Sirtema
Blondy	Hercules	Pallina	Sjamero
Bonanza		Palma	Solara
	Impala	Panda	Sommergold
Calgary	Indira	Patrona	Stabilo
Calla	Irmgard	Pepo	
Christa		Petra	Taiga
Cilena	Jetta	Pia	Tempora
Cinja	Juvena	Planta	Thomana
Colette		Ponto	Tomba
Combi	Kanjer	Premiere	Tomensa
	Karakter	Producent	
Darwina	Karatop	Provita	Ukama
Desiree	Kardal	Prudenta	Ulla
Dinia	Karida		Ute
Ditta	Kardent	Quarta	
Donald	Karlena	Quinta	Vebeca
Donella	Karnico		Velox
	Kennebec	Rebecca	Vinetta
Eersteling	Kolibri	Renate	
Elkana	Kuras	Rex	Walli
Elles		Rikea	Wilja

Bioassay

Samples positive in IF- and/or PCR-tests were further tested by bioassay in eggplant seedlings (cv. Black Beauty) according to the standard EC-protocol (Anonymous, 1993). Plants were grown in a computer-controlled glasshouse at 23 °C. Eggplants were examined for symptoms over 40 days. From eggplants showing symptoms, isolation, identification and confirmation of the causal organism was performed according to the standard EC-protocol (Anonymous, 1993). If eggplants showed no symptoms after 40 days, approximately 2-cm stem pieces were removed just above the inoculation site and surface-disinfected with 70% ethanol. Sample preparation was performed by the procedure described above and the homogenates were tested by IF and PCR. Samples positive in IF- and/or PCR-tests were further tested in a second eggplant-bioassay for 40 days. If symptoms were not observed in the second bioassay, the samples were considered negative according to the standard EC-protocol (Anonymous, 1993). Additionally, however, homogenates of symptomless eggplants after the second bioassay were further tested with IF- and PCR. Samples which tested positive in IF- and/or PCR-tests were re-tested in up to 3 further bioassays until symptoms appeared in the eggplants.

Results

In the multiplex PCR, the primer sets PSA-1/PSA-R (*C. michiganensis* subsp. *sepedonicus*-specific) and NS-7-F/NS-8-R (plant-specific) were used simultaneously in the same reaction mixture. The reaction conditions of the multiplex PCR were carefully optimized with respect to yield of the different PCR products and detection sensitivity for *C. michiganensis* subsp. *sepedonicus*. The optimization experiments were performed with DNA extracts from potato tuber samples artificially inoculated with a dilution series of *C. michiganensis* subsp. *sepedonicus* cells. The relative concentrations of the primer sets PSA-1/PSA-R and Ns-7-F/Ns-8-R were critical for the sensitivity of the *C. michiganensis* subsp. *sepedonicus* PCR assay. Several combinations of primer concentrations were tested and the detection sensitivity was compared with the primer set PSA-1/PSA-R in 'uniplex PCR' (Figure 1). The highest sensitivity was achieved with 0.2 μM of primers PSA-1/PSA-R and 0.04 μM of primers NS-7-F/NS-8-R. Using higher concentrations of primer set NS-7-F/NS-8-R resulted in increased amplification of the plant-specific DNA fragment, but decreased detection sensitivity for *C. michiganensis* subsp. *sepedonicus* (data not shown).

Using the optimized multiplex PCR protocol, it was possible to detect artificially added *C. michiganensis* subsp. *sepedonicus* in potato core fluid in the range of 2–20 CFU per PCR reaction mixture (20–200 CFU per ml potato core fluid) (Figure 2, lanes 6 and 7). This is equivalent to the detection sensitivity of the 'uniplex PCR' using primer set PSA-1/PSA-R (Figure 1). Multiplex PCR with DNA extracts of potato tubers infected with *C. michiganensis* subsp. *sepedonicus* generated 2 discrete DNA fragments in the size of 502 bp (*C. michiganensis* subsp. *sepedonicus*-specific) and 377 bp (plant-specific) (Figure 2, lanes 1–7). Samples

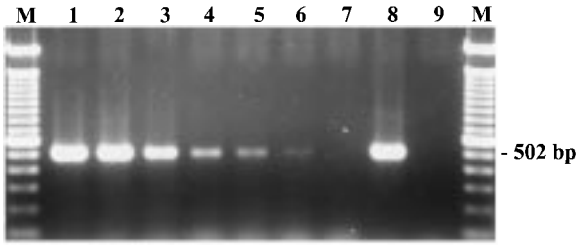

Figure 1. Sensitivity of the 'uniplex' PCR for *C. michiganensis* subsp. *sepedonicus* in potato core fluid employing the *C. michiganensis* subsp. *sepedonicus*-specific primer set PSA-1/PSA-R. Healthy potato tuber extracts were mixed with 10-fold dilutions of *C. michiganensis* subsp. *sepedonicus* cells. Lanes 1–7, dilutions of *C. michiganensis* subsp. *sepedonicus* cells ranging from 2×10^5 to 0.2 CFU per PCR reaction mixture; lane 8, genomic DNA of *C. michiganensis* subsp. *sepedonicus* (GSPB 1522); lane 9, negative control; lanes M, DNA size marker (100 bp ladder, Life Technologies, Germany).

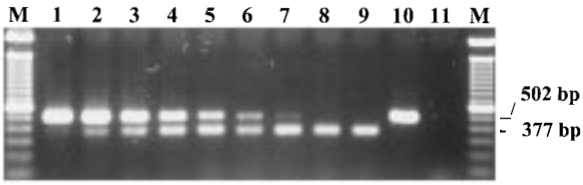

Figure 2. Sensitivity of the multiplex PCR for *C. michiganensis* subsp. *sepedonicus* in potato core fluid. Healthy potato tuber extracts were mixed with 10-fold dilutions of *C. michiganensis* subsp. *sepedonicus* cells. Lanes 1–8, dilutions of *C. michiganensis* subsp. *sepedonicus* cells ranging from 2×10^6 to 0.2 CFU per PCR reaction mixture; lane 9, healthy potato sample; lane 10, genomic DNA of *C. michiganensis* subsp. *sepedonicus* (GSPB 1522); lane 11, negative control; lanes M, DNA size marker (100 bp ladder, Life Technologies, Germany).

with increasing amounts of bacteria resulted in a stronger signal intensity of the *C. michiganensis* subsp. *sepedonicus*-specific PCR product, but decreased amplification of the plant-specific DNA fragment. Samples of potato core fluid without *C. michiganensis* subsp. *sepedonicus* only amplified the plant-specific fragment in the size of 377 bp (Figure 2, lanes 8 and 9). Multiplex PCR with genomic DNA extracts of *C. michiganensis* subsp. *sepedonicus* produced only the *C. michiganensis* subsp. *sepedonicus*-specific DNA fragment and no plant-specific DNA fragment, due to the absence of plant DNA in the bacterial genomic DNA extracts (Figure 2, lane 10).

Different Taq DNA polymerases were tested for their use in multiplex PCR. Not all these polymerases amplified both targets in the same PCR reaction (data not shown). Consistently reliable results were obtained with 'Platinum' Taq DNA polymerase (Life Technologies, Germany), which is a 'hot-start' Taq DNA polymerase. The activity of this enzyme is blocked at ambient temperature but is regained after the denaturation step in PCR cycling. This potentially reduces non-specific amplification in PCR. Nevertheless, comparable results were obtained employing the native Taq DNA polymerase (Life Technologies, Germany) (data not shown). However, with both of these enzymes, sometimes an additional weak PCR product of 650 bp was observed, which was easily discernible on agarose gel (Figure 3, lanes 2, 3 and 7, 8). This DNA fragment appeared independent of the tested potato cultivar and was probably due to non-specific 'background' amplification of unknown origin.

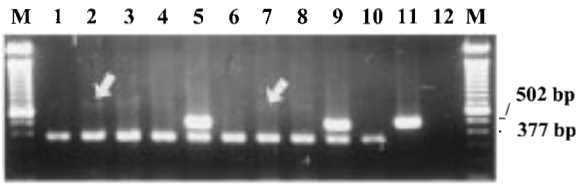

Figure 3. Representative multiplex PCR results in surveys for *C. michiganensis* subsp. *sepedonicus* in potato seed samples. Lanes 1–8, DNA of various potato seed samples; lane 9, DNA of potato extracts mixed with *C. michiganensis* subsp. *sepedonicus* cells (2×10^5); lane 10, DNA of potato extracts mixed with *Ralstonia solanacearum* cells (2×10^5); lane 11, genomic DNA of *C. michiganensis* subsp. *sepedonicus* (GSPB 1522); lane 12, negative control; lanes M, DNA size marker (100 bp ladder, Life Technologies, Germany). Arrows indicate 'background' amplification.

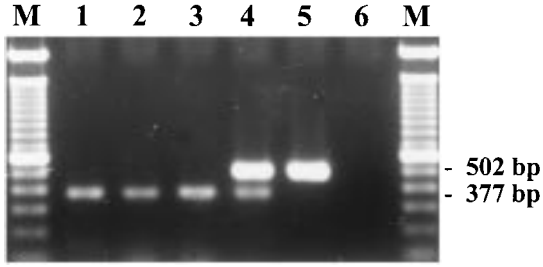

Figure 4. Multiplex PCR with various DNA extracts as template. Lane 1, DNA of tomato (cv. Moneymaker); lane 2, DNA of eggplant (cv. Black Beauty); lane 3, DNA of potato; lane 4, DNA of potato extracts mixed with *C. michiganensis* subsp. *sepedonicus* cells (2×10^5); lane 5, genomic DNA of *C. michiganensis* subsp. *sepedonicus* (GSPB 1522); lane 6, negative control; lanes M, DNA size marker (100 bp ladder, Life Technologies, Germany).

To test the specificity of the multiplex PCR, amplification was carried out with genomic DNA of the bacterial strains listed in Table 1 and DNA of tomato (cv. Moneymaker), eggplant (cv. Black Beauty) and potato. The amplified DNA fragment of 502 bp was specifically obtained with strains of *C. michiganensis* subsp. *sepedonicus* (Figure 4, lane 5). No amplification products were observed with related subspecies or with other bacteria tested (data not shown). PCR with DNA of tomato, eggplant and potato generated a 'universal' amplicon of 377 bp (Figure 4, lanes 1–3).

The specificity of the multiplex PCR was confirmed by restriction analysis of the amplified products with *Bgl*II, whose restriction site is present in the *C. michiganensis* subsp. *sepedonicus*-specific PCR product. The restriction fragments obtained from the *C. michiganensis* subsp. *sepedonicus*-specific DNA fragment were 282 and 220 bp in size (Figure 5, lanes 1 and 2). No restriction site was present in the plant-specific amplicon (Figure 5, lanes 5 and 6). Restriction analysis of multiplex PCR products of a *C. michiganensis* subsp. *sepedonicus*-positive potato sample resulted in 3 DNA fragments, 377, 282 and 220 bp in size (Figure 5, lanes 3 and 4).

Approximately 3500 composite potato tuber samples of 143 different cultivars (Table 3) were tested for *C. michiganensis* subsp. *sepedonicus* in parallel using the IF-assay and multiplex PCR. In most cases (99%) the samples tested negative with IF and multiplex PCR. Out of approximately 3500 composite samples tested, 36 were positive in IF-assay and/or multiplex PCR (Table 4). Eleven samples were positive in IF

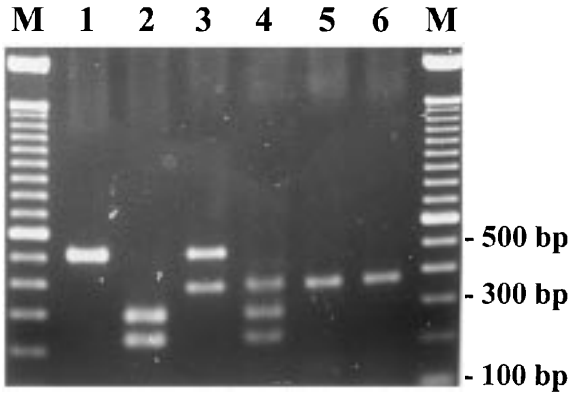

Figure 5. Restriction analysis of multiplex PCR products with *Bgl*II (Boehringer Mannheim, Germany). Lanes 1 and 2, undigested and digested *C. michiganensis* subsp. *sepedonicus*-specific PCR product; lanes 3 and 4, undigested and digested multiplex PCR products of a *C. michiganensis* subsp. *sepedonicus*-positive 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).

(both antisera), multiplex PCR and bioassay (Table 4, Nos. 1–11). Seven samples were negative in IF (both antisera), but positive in multiplex PCR and positive in the bioassay (Table 4, Nos. 12–18). Seven samples were positive in IF (both antisera) and multiplex PCR, but were negative in the bioassay (Table 4, Nos. 19–25). Eight samples were negative in IF (both antisera), but positive in multiplex PCR and negative in the bioassay (Table 4, Nos. 26–33). Three samples were positive with the polyclonal antiserum, but negative with the monoclonal antiserum, the multiplex PCR and the bioassay. Among 143 different potato cultivars tested (Table 3), all amplified the expected universal PCR product of 377 bp (data not shown).

Discussion

The main objective was the development of a reliable screening PCR assay for the detection of the pathogen *C. michiganensis* subsp. *sepedonicus* in potato tubers. The new PCR assay should combine two different tests in one reaction mixture. First, a highly specific and sensitive detection of the pathogen, second, an indicator test for successful amplification (internal PCR control), which monitors potentially false-negative PCR results, generated by reaction failure of the PCR.

Therefore, a multiplex PCR assay (Chamberlain et al., 1988) was developed, which amplified simultaneously two different targets in the same PCR reaction mixture by two different primer sets. For the detection of *C. michiganensis* subsp. *sepedonicus*, the pathogen-specific primer set PSA-1/PSA-R (Pastrik and Rainey, 1999) was used and for the amplification of the internal PCR control, the plant-specific primer set NS-7-F/NS-8-R (Dams et al., 1988; White et al., 1990) was employed. The plant-specific primers were able to amplify the predicted 'universal' PCR fragment in the size of 377 bp from DNA of potato, tomato (cv. Moneymaker) and eggplant (cv. Black Beauty). The eggplant is used in the bioassay for detection of *C. michiganensis* subsp. *sepedonicus* (Anonymous, 1993). The application of this target sequence for the internal PCR control was possible, because plant DNA was present in DNA from potato extracts.

The use of an internal positive control was previously described by Hu et al. (1995). However, this assay does not indicate the success of DNA isolation and lacks specificity to *C. michiganensis* subsp. *sepedonicus* (Drennan et al., 1993).

To establish a robust two-amplicon multiplex PCR system, it was necessary to optimize reaction components and cycling conditions very carefully (Chamberlain et al., 1991). Optimization of the primer concentrations was important with respect to the PCR product yield of the different targets and the detection sensitivity for *C. michiganensis* subsp. *sepedonicus*. The sensitivity of the detection system was impaired at high concentrations of the plant-specific primer set, resulting in increased amplification of the plant-specific DNA fragment, but decreased detection sensitivity for *C. michiganensis* subsp. *sepedonicus*. This effect of primer ratio on signal intensity probably reflects a form of competitive PCR described by Hu et al. (1995). The simultaneous amplification of two different targets in one reaction mixture was not associated with a lower sensitivity of the assay. The detection level of the optimized multiplex PCR was in the range of 2–20 CFU per PCR reaction mixture which is equivalent to that found in 'uniplex' PCR protocols (Lee et al., 1997; Li and DeBoer, 1995; Mills et al., 1997; Pastrik and Rainey, 1999). As with most PCR reactions, equivalent sensitivity of detection under different laboratory conditions is likely to require additional optimization of reaction conditions.

Table 4. List of the 36 out of 3500 composite samples of seed potato tubers having at least one positive result in IF, PCR or bioassay

Sample No.	IF-test[a] (polyclonal)	IF-test[a] (monoclonal)	PCR-test[b]	Bioassay[c]
1	pos (2.9×10^8)	pos (2.9×10^8)	pos (+++)	pos (1)
2	pos (2.3×10^8)	pos (2.3×10^8)	pos (+++)	pos (1)
3	pos (1.6×10^8)	pos (1.5×10^8)	pos (+++)	pos (1)
4	pos (1.5×10^8)	pos (1.5×10^8)	pos (+++)	pos (1)
5	pos (4.9×10^7)	pos (4.9×10^7)	pos (+++)	pos (1)
6	pos (4.2×10^7)	pos (4.2×10^7)	pos (+++)	pos (1)
7	pos (3.9×10^7)	pos (4.2×10^7)	pos (+++)	pos (2)
8	pos (2.1×10^7)	pos (2.0×10^7)	pos (+++)	pos (1)
9	pos (6.4×10^6)	pos (4.9×10^6)	pos (+++)	pos (2)
10	pos (6.2×10^6)	pos (6.7×10^6)	pos (++)	pos (1)
11	pos (9.8×10^5)	pos (9.1×10^5)	pos (++)	pos (2)
12	neg (6.5×10^4)	neg (4.9×10^4)	pos (+)	pos (3)
13	neg (4.6×10^4)	neg (2.9×10^4)	pos (+)	pos (4)
14	neg (2.6×10^4)	neg (2.6×10^4)	pos (++)	pos (3)
15	neg (1.6×10^4)	neg (1.3×10^4)	pos (++)	pos (4)
16	neg (0)	neg (0)	pos (++)	pos (4)
17	neg (0)	neg (0)	pos (+)	pos (5)
18	neg (0)	neg (0)	pos (++)	pos (5)
19	pos (1.3×10^8)	pos (1.3×10^8)	pos (+++)	neg (2)
20	pos (2.6×10^7)	pos (2.7×10^7)	pos (+++)	neg (1)
21	pos (3.8×10^6)	pos (4.1×10^6)	pos (+++)	neg (2)
22	pos (1.1×10^6)	pos (8.2×10^5)	pos (++)	neg (2)
23	pos (6.5×10^5)	pos (7.2×10^5)	pos (++)	neg (1)
24	pos (5.9×10^5)	pos (6.2×10^5)	pos (++)	neg (2)
25	pos (3.6×10^5)	pos (2.6×10^5)	pos (++)	neg (1)
26	neg (7.5×10^4)	neg (7.8×10^4)	pos (++)	neg (2)
27	neg (4.6×10^4)	neg (5.9×10^4)	pos (+)	neg (1)
28	neg (4.6×10^4)	neg (1.3×10^4)	pos (++)	neg (2)
29	neg (3.6×10^4)	neg (4.2×10^4)	pos (+)	neg (1)
30	neg (3.3×10^4)	neg (0)	pos (++)	neg (2)
31	neg (3.3×10^2)	neg (1.6×10^3)	pos (++)	neg (1)
32	neg (0)	neg (0)	pos (++)	neg (1)
33	neg (0)	neg (0)	pos (+)	neg (1)
34	pos (2.1×10^6)	neg (0)	neg	neg (1)
35	pos (7.2×10^5)	neg (0)	neg	neg (1)
36	pos (6.2×10^5)	neg (0)	neg	neg (1)

[a]Threshold > 3 IFU per microscope field $= 1 \times 10^5$ cells per ml of resuspended pellet, positive.
[b]Intensity of the amplified *C. michiganensis* subsp. *sepedonicus*-specific fragment: + = weak; ++ = medium; +++ = strong band.
[c]neg: no symptoms and negative IF/PCR-test; pos: symptoms; numbers indicate the number of times the bioassay was repeated until a positive or negative result was obtained.

Furthermore, it was important to standardize the method of sample preparation from potato tubers. Different procedures have been published (Anonymous, 1993). Depending on the method, different concentrations of plant DNA, which is the target sequence for the internal PCR control, may be obtained. For example with the 'shaking' method of sample preparation (Dinesen, 1984), which was not tested in this study, the removed tissue cores of the tubers are only incubated in buffer and not crushed. The multiplex PCR described in this study was standardized for DNA extracts from potato samples prepared with a maceration procedure, by which all 200 tissue cores in a sample were completely homogenized.

To verify the applicability of the multiplex PCR described in this paper, the method was employed in surveys for *C. michiganensis* subsp. *sepedonicus* in potato seed samples. The threshold of detection by IF-assay was set at 1×10^5 cells per ml of resuspended pellet. Although it is recognized that the presence of any fluorescing cells potentially indicates the presence of *C. michiganensis* subsp. *sepedonicus*, the occasional presence of cross-reacting bacteria requires the setting of a threshold level (DeBoer et al., 1994; 1996; Samson et al., 1987). Nevertheless, samples with bacterial concentrations estimated to be lower than this threshold were tested in the bioassay in order to fulfil the standard EC-protocol for the detection and diagnosis of the ring rot bacterium in potato tubers (Anonymous, 1993). It should be noted that higher threshold levels than this used in this study have been established elsewhere (DeBoer et al., 1996).

In most cases the potato seed samples tested negative with both IF and multiplex PCR. A good correlation of the results were observed with 11 potato seed samples, which tested positive with IF, multiplex PCR and bioassay. For these samples the estimated number of IFU (N) per ml of resuspended pellet ranged from 9.8×10^5 to 2.9×10^8.

However, with multiplex PCR it was possible to confirm the presence of *C. michiganensis* subsp. *sepedonicus* in 7 potato samples in which fluorescent cells were not observed in IF (in 3 cases) or where observed in numbers below the threshold of 1×10^5 cells per ml. In all 7 of these cases it was eventually possible to obtain a positive bioassay result (although only after repeating this test up to 5 times), indicating a false-negative IF result. Positive results by multiplex PCR, were attributed to the higher sensitivity of this test.

Seven potato samples tested positive with IF (both antisera) and multiplex PCR, but tested negative in the bioassay, possibly due to detection of non-viable or avirulent cells by PCR and IF. Other parameters known to affect sensitivity of the bioassay are concentration of plant debris in samples taken from infected tubers (Zeller and Xie, 1985), the growth conditions of the eggplants (Janse and Van Vaerenbergh, 1987), virulence variation of *C. michiganensis* subsp. *sepedonicus* strains (Bishop and Slack, 1987) and suppression of *C. michiganensis* subsp. *sepedonicus* by antagonistic bacteria (Gamard and DeBoer, 1995).

Eight potato samples tested positive with multiplex PCR, but negative with IF (both antisera) and bioassay, although in 6 of them fluorescing cells were observed in IF at populations lower than the threshold. It was not possible to determine whether these results were due to cross-reaction in both PCR and IF, or due to the inoculum concentration being below the borderline for detection by the bioassay (Janse and Van Vaerenbergh, 1987).

The specificity of the multiplex PCR was demonstrated by the discrimination of three 'false-positive' IF results obtained with the polyclonal antiserum, attributed to cross-reactions with other bacteria (Bishop et al., 1988; Calzolari et al., 1982; DeBoer, 1982). These 3 potato samples tested negative in subsequent IF with the monoclonal antibody, the multiplex PCR and the bioassay.

Inhibition of PCR by plant compounds in crude extracts from the potato cultivar Desiree has been reported (Elphinstone et al., 1996). In our surveys, 143 different cultivars were tested, among them several samples were of cultivar Desiree. With all of these cultivars, the universal amplicon in the size of 377 bp was obtained, indicating that no inhibition of PCR reactions had occurred. However, PCR failure was observed with samples of potato tubers with adherent soil, due to inhibitory effects of humic acids (Abbaszadegan et al., 1993; Tsai et al., 1993; Tsai and Olsen, 1992). To circumvent these inhibitory effects, all potato samples were washed before removing the tissue cores from the stolon ends.

In conclusion, the multiplex PCR protocol described here is sensitive and specific for the detection of *C. michiganensis* subsp. *sepedonicus* in potato tubers. It provides a useful tool to monitor the occurrence of *C. michiganensis* subsp. *sepedonicus* in potato samples and to manage the availability of pathogen-free potato seed tubers.

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References

Abbaszadegan M, Huber MS, Gerba CP and Pepper IL (1993) Detection of enteroviruses in groundwater with the polymerase chain reaction. Appl Environ Microbiol 59: 1318–1324

Anonymous (1993) Council Directive 93/85/EEC of 4 October 1993 on the control of potato ring rot. Official Journal of the European Communities, No. L259/1, 18.10.93, Annex I–IV

Anonymous (1977) Council Directive 77/93/EEC of 21 December 1976 on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community. Official Journal of the European Communities, No. L26, 31.01.1977

Bej AK, McCarty SC and Atlas RM (1991) Detection of coliform bacteria and *Escherichia coli* by multiplex polymerase chain reaction: comparison with defined substrate and plating methods for water quality monitoring. Appl Environ Microbiol 57: 2429–2432

Bej AK, Mahbubani MH, Miller R, DiCesare JL, Haff L and Atlas RM (1990) Multiplex PCR amplification and immobilized capture probes for detection of bacterial pathogens and indicators in water. Molecul Cell Probes 4: 353–365

Bishop AL, Clarke RG and Slack SA (1988) Antigenic anomaly in a naturally occuring nonfluidal strain of *Corynebacterium sepedonicum*. Am Potato J 65: 237–244

Bishop AL and Slack SA (1987) Effect of inoculum dose and preparation, strain variation, and plant growth conditions on the eggplant assay for bacterial ring rot. Am Potato J 64: 227–234

Calzolari A, Bazzi C and Mazzucchi U (1982) Cross reactions between *Corynebacterium sepedonicum* and *Arthrobacter polychromogenes* in immunofluorescence staining. Potato Research 25: 239–246

Chamberlain JS, Gibbs RA, Ranier JE and Caskey CT (1991) Detection of gene deletions using multiplex polymerase chain reactions. In: Mathew C (ed) Methods in Molecular Biology: Protocols in Human Molecular Genetics, Vol 9 (pp 299–312) Humana Press, Clifton, New Jersey

Chamberlain JS, Gibbs RA, Ranier JE, Ngyen PN and Caskey CT (1988) Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification. Nucl Acids Res 16: 11141–11156

Dams EL, Hendriks L, Van de Peer Y, Neefs JM, Smits G, Vandenbempt I and De Wachter R (1988) Compilation of small ribosomal subunit RNA sequences. Nucl Acids Res 16(Suppl): r87–r173

DeBoer SH (1982) Cross-reaction of *Corynebacterium sepedonicum* antisera with *C. insidiosum, C. michiganense* and an unidentified coryneform bacterium. Phytopathology 72: 1474–1478

DeBoer SH and McNaughton ME (1986) Evaluation of immunofluorescence with monoclonal antibodies for detection of latent bacterial ring rot infection. Am Potato J 63: 533–543

DeBoer SH, Wieczorek A and Kummer A (1988) An ELISA test for bacterial ring rot of potato with a new monoclonal antibody. Plant Disease 72: 874–878

DeBoer SH, Stead DE, Alivizatos AS, Janse JD, Van Vaerenbergh J, De Haan TL and Mawhinney J (1994) Evaluation of serological tests for detection of *Clavibacter michiganensis* subsp. *sepedonicus* in composite potato stem and tuber samples. Plant Disease 78: 725–729

DeBoer SH, Boucher A and De Haan TL (1996) Validation of thresholds for serological tests that detect *Clavibacter michiganensis* subsp. *sepedonicus* in potato tuber tissue. EPPO Bull/Bull OEPP 26: 391–398

Dinesen IG (1984) The extraction and diagnosis of *Corynebacterium sepedonicum* from diseased potato tubers. EPPO Bull/Bull OEPP 14(2): 147–152

Drennan JL, Westra AAG, Slack SA, Delserone LM and Collmer A (1993) Comparison of a DNA hybridization probe and ELISA for the detection of *Clavibacter michiganensis* subsp. *sepedonicus* in field-grown potatoes. Plant Disease 77: 1243–1247

Elphinstone JG, Hennessy J, Wilson JK and Stead DE (1996) Sensitivity of different methods for the detection of *Ralstonia solanacearum* in potato tuber extracts. EPPO Bull/Bull OEPP 26: 663–678

Franken AAJM, Kamminga GC, Snijders W, van der Zouwen PS and Birnbaum YE (1993) Detection of *Clavibacter michiganensis* ssp. *michiganensis* in tomato seeds by immunoflourescence microscopy and dilution plating. Neth J Plant Pathol 99: 125–137

Gamard P and DeBoer SH (1995) Evaluation of antagonistic bacteria for suppression of bacterial ring rot of potato. Europ J Plant Pathol 101: 519–525

Hu X, Lai F-M, Reddy ASN and Ishimaru CA (1995) Quantitative detection of *Clavibacter michiganensis* subsp. *sepedonicus* by competitive polymerase chain reaction. Phytopathology 85: 1468–1473

Janse JD and Van Vaerenbergh J (1987) Interpretation of the EC method for the detection of latent *Corynebacterium sepedonicum* infections in potato. EPPO Bull/Bull OEPP 17: 1–10

Lee IM, Bartoszyk IM, Gundersen DE, Mogen B and Davis RE (1997) Nested PCR for ultrasensitive detection of the potato ring rot bacterium, *Clavibacter michiganensis* subsp. *sepedonicus*. Appl Environ Microbiol 63: 2625–2630

Li X and DeBoer SH (1995) Selection of polymerase chain reaction primers from an RNA intergenic spacer region for specific detection of *Clavibacter michiganensis* subsp. *sepedonicus*. Phytopathology 85: 837–842

Mansfield-Giese K (1997) Plant-to-plant transmission of the bacterial ring rot pathogen *Clavibacter michiganensis* subsp. *sepedonicus*. Potato Research 40: 229–235

Mogen BD and Oleson AE (1987) Homology of pCS1 plasmid with chromosomal DNA in *Clavibacter michiganensis* subsp. *sepedonicus*: Evidence for the presence of a repeated sequence and plasmid integration. Appl Environ Microbiol 53: 2476–2481

Mogen BD, Oleson AE, Sparks RB, Gudmestad NC and Secor GA (1988) Distribution and partial characterization of pCS1, a highly conserved plasmid present in *Clavibacter michiganense* subsp. *sepedonicum*. Phytopathology 78: 1381–1386

Mills D, Russell BW and Hanus JW (1997) Specific detection of *Clavibacter michiganensis* subsp. *sepedonicus* by amplification of three unique DNA sequences isolated by southern hybridization. Phytopathology 87: 853–861

Nelson GA (1980) Long-term survival of *Corynebacterium sepedonicum* on contaminated surfaces and in infected potato stems. Am Potato J 57: 595–600

Pastrik KH and Rainey FA (1999) Identification and differentiation of *Clavibacter michiganensis* subspecies by PCR-based techniques. J Phytopathol 147: 687–693

Powell HA, Gooding CM, Garrett SD, Lund BM and McKee RA (1994) Proteinase inhibition of the detection of *Listeria monocytogenes* in milk using the polymerase chain reaction. Lett Appl Microbiol 18: 59–61

Rhodes ME (1959) The characterisation of *Pseudomonas fluorescens*. J Gen Microbiol 21: 221–263

Rodriguez ER, Nasim S, Hsia J, Sandin RL, Ferreira A, Hilliard BA, Ross AM and Garret CT (1991) Cardiac myocytes and dendritic cells harbor human immunodeficiency virus in infected patients with and without cardiac dysfunction: detection by multiplex, nested, polymerase chain reaction in individually microdissected cells from right ventricular endomyocardial biopsy tissue. Am J Cardiol 68: 1511–1520

Rossen L, Norskov P, Holmstrom K and Rasmussen OF (1992) Inhibition of PCR by components of food samples, microbial diagnostic assays and DNA-extraction solutions. Int J Food Microbiol 17: 37–45

Samson R, Poutier F and Bregon A (1987) Establishment of positive and negative thresholds for immunofluorescent detection of bacteria, with special reference to *Corynebacterium sepedonicum* in potato tubers. EPPO Bull/Bull OEPP 17: 300–301

Schneider BJ, Zhao J and Orser CS (1993) Detection of *Clavibacter michiganensis* subsp. *sepedonicus* by DNA amplification. FEMS Microbiol Lett 109: 207–212

Tsai YL and Olsen BH (1992) Detection of low numbers of bacterial cells in soils and sediments by polymerase chain reaction. Appl Environ Microbiol 58: 754–757

Tsai Y, Palmer CJ and Sangerman G (1993) Detection of *Escherichia coli* in sewage and sludge by polymerase chain reaction. Appl Environ Microbiol 59: 353–357

Way JS, Josephson KL, Pillai SD, Abbaszadegan M, Gerba CP and Pepper IL (1993) Specific detection of *Salmonella* spp. by multiplex polymerase chain reaction. Appl Environ Microbiol 59: 1473–1479

White TJ, Bruns T, Lee S and Taylor J (1990) Amplification and direct sequencing of fungal ribosomal RNA genes for phylogenetics. In: Innis MA, Gelfand DH, Sninsky JJ and White TJ (eds) PCR Protocols: A Guide to Methods and Applications (pp 315–322) Academic Press, Inc

Zeller W and Xie Y (1985) Studies of diagnosis of bacterial ring rot of potatoes, I. Pathogenicity test in eggplants. Phytopathol Z 112: 198–206

Zielke R and Naumann K (1984) Untersuchungen zur Erfassung des latenten Befallsstadiums von *Corynebacterium sepedonicum* im Kartoffelgewebe. Zbl Mikrobiol 139: 267–280