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A Quantitative and Direct PCR Assay for the Subspecies-Specific Detection of *Clavibacter michiganensis* subsp. *michiganensis* Based on a Ferredoxin Reductase Gene

Min Seok Cho[1], Jang Ha Lee[2], Nam Han Her[2], ChangKug Kim[1], Young-Joo Seol[1], Jang Ho Hahn[1], Ji Hyoun Baeg[3], Hong Gi Kim[4], and Dong Suk Park[1*]

[1]*National Academy of Agricultural Science, Rural Development Administration, Suwon 441-707, Republic of Korea*
[2]*Nongwoo Bio, Suwon 443-372, Republic of Korea*
[3]*Animal, Plant and Fisheries Quarantine and Inspection Agency, Incheon International Airport Regional Office, Baggage Quarantine and Inspection Division, Incheon 400-718, Republic of Korea*
[4]*Department of Applied Biology, Chungnam National University, Daejeon 305-764, Republic of Korea*

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The Gram-positive bacterium *Clavibacter michiganensis* subsp. *michiganensis* is the causal agent of canker disease in tomato. Because it is very important to control newly introduced inoculum sources from commercial materials, the specific detection of this pathogen in seeds and seedlings is essential for effective disease control. In this study, a novel and efficient assay for the detection and quantitation of *C. michiganensis* subsp. *michiganensis* in symptomless tomato and red pepper seeds was developed. A pair of polymerase chain reaction (PCR) primers (Cmm141F/R) was designed to amplify a specific 141 bp fragment on the basis of a ferredoxin reductase gene of *C. michiganensis* subsp. *michiganensis* NCPPB 382. The specificity of the primer set was evaluated using purified DNA from 16 isolates of five *C. michiganensis* subspecies, one other *Clavibacter* species, and 17 other reference bacteria. The primer set amplified a single band of expected size from the genomic DNA obtained from the *C. michiganensis* subsp. *michiganensis* strains but not from the other *C. michiganensis* subspecies or from other *Clavibacter* species. The detection limit was a single cloned copy of the ferredoxin reductase gene of *C. michiganensis* subsp. *michiganensis*. In conclusion, this quantitative direct PCR assay can be applied as a practical diagnostic method for epidemiological research and the sanitary management of seeds and seedlings with a low level or latent infection of *C. michiganensis* subsp. *michiganensis*.

Keywords**:** *Clavibacter michiganensis* subsp. *michiganensis*, detection, quantitation, tomato, ferredoxin reductase

*For correspondence. E-mail: dspark@rda.go.kr; Tel.: +82-31-299-1669; Fax: +82-31-299-1652

Introduction

Bacterial canker of tomato (*Lycopersicon esculentum* Mill.) is caused by *Clavibacter michiganensis* subsp. *michiganensis* and is one of the most destructive diseases of tomato (Fatmi and Schaad, 2002). It is also the cause of crop failure in all of the main cultivation areas because neither resistant tomato cultivars nor effective chemical controls of this pathogen are available (Dreier *et al.*, 1995).

The control of this disease is particularly important for tomato seed producers because infected seeds are considered the primary inoculum source and current legislation forbids their commercialization (Leon *et al.*, 2006). Seed dissemination is the major cause for many of the new outbreaks of bacterial canker and its worldwide distribution, and the most important strategy for controlling bacterial canker has been the use of pathogen-free seeds (Rademaker and Janse, 1994; Janse and Wenneker, 2002). Therefore, there is great demand for reliable diagnostic methods to detect this important pathogen.

The conventional methods used for the detection and identification of this pathogen include biochemical tests, serological assays, and fatty acid and metabolic profiling. These methods, however, have several limitations, including a poor sensitivity, lack of specificity and a long waiting period for results (Leon *et al.*, 2006).

Currently, molecular assays based on 16S–23S rDNA spacer region are widely used for the detection of *C. michiganensis* subsp. *michiganensis*; although the region was amplified only from strains of *C. michiganensis* subsp. *michiganensis*, the sequence differed at only two or three bases with respect to the analogous regions in two closely related subspecies. Therefore, this technique requires more strict control of the annealing temperature to maintain primer specificity (Bach *et al.*, 2003; Zhao *et al.*, 2007).

To our knowledge, there are not many other techniques that have been developed in recent years for the detection of *C. michiganensis* subsp. *michiganensis*; thus, the most sensitive procedure is remains the isolation of the microorganism using semiselective media.

In this study, therefore, a subspecies-specific primer set based on the ferredoxin reductase gene of *C. michiganensis* subsp. *michiganensis* was designed to develop a highly sensitive and specific method that could aid in the detection of *C. michiganensis* subsp. *michiganensis* in infected tomato seed lots.

It is known that ferredoxin reductase is a member of the flavoprotein pyridine nucleotide cytochrome reductases

(FPNCR) that catalyze the interchange of reducing equivalents between one-electron carriers and the two-electron-carrying nicotinamide dinucleotides (Hyde *et al.*, 1991). Ferredoxin reductase catalyzes the final step of electron transfer to generate nicotinamide adenine dinucleotide phosphate (NADPH) and ATP in plant chloroplasts during photosynthesis. However, FPNCRs show no sequence similarity to NADPH:adrenodoxin reductases or, to bacterial ferredoxin: NAD reductases and their homologs (http://www.ebi.ac.uk/interpro/IEntry?ac=IPR015701) despite their functional similarities (Hanukoglu and Gutfinger, 1989).

This report describes the design of specific PCR primers that distinguish *C. michiganensis* subsp. *michiganensis* from *Clavibacter michiganensis*, and the optimization of protocols for the detection of *C. michiganensis* from tomato seeds and seedlings.

Materials and Methods

Bacterial strains and culture conditions

All of the bacterial strains were obtained from the Belgian Coordinated Collections of Micro-organisms (BCCM), the Korean Agricultural Culture Collection (KACC) in the Republic of Korea, and the National Collection of Plant Pathogenic Bacteria (NCPPB) in the United Kingdom. The bacterial strains used in this study are listed in Table 1. The following culture media and incubation conditions were

Table 1. Bacterial strains used in the PCR specificity test

No.	Bacterial strains[a]	Source	Geographical origin	*Pat*-1 (Dreier *et al.*, 1995)	16S–23S rRNA (Pastrik and Rainey, 1999)	Ferredoxin reductase gene[b] (this study)
1	*Clavibacter michiganensis* subsp. *michiganansis*[T]	LMG 7333	Hungary	+[c]	+	+
2	*Clavibacter michiganensis* subsp. *michiganansis*	LMG 3681	United Kingdom	+	+	+
3	*Clavibacter michiganensis* subsp. *michiganansis*	LMG 3679	Kenya	+	+	+
4	*Clavibacter michiganensis* subsp. *michiganansis*	LMG 3685	United States	-	+	+
5	*Clavibacter michiganensis* subsp. *michiganansis*	LMG 5602	New Zealand	-	+	+
6	*Clavibacter michiganensis* subsp. *michiganansis*	LMG 5643	Canada	+	+	+
7	*Clavibacter michiganensis* subsp. *michiganansis*	LMG 5725	Bulgaria	+	+	+
8	*Clavibacter michiganensis* subsp. *michiganansis*	LMG 5726	United States	+	+	+
9	*Clavibacter michiganensis* subsp. *michiganansis*	LMG 5727	Bulgaria	+	+	+
10	*Clavibacter michiganensis* subsp. *michiganansis*	LMG 5728	Bulgaria	+	+	+
11	*Clavibacter michiganensis* subsp. *sepedonicus*[T]	LMG 2889	Canada	-	+	-
12	*Clavibacter michiganensis* subsp. *sepedonicus*	LMG 2897	United States	-	+	-
13	*Clavibacter michiganensis* subsp. *sepedonicus*	LMG 5861	Sweden	-	-	-
14	*Clavibacter michiganensis* subsp. *insidiosus*[T]	LMG 3663	United States	-	+	-
15	*Clavibacter michiganensis* subsp. *nebraskensis*[T]	LMG 3700	United States	-	+	-
16	*Clavibacter michiganensis* subsp. *tesselarius*[T]	NCPPB 3664	United States	-	+	-
17	*Clavibacter rathayi*[T]	LMG 7288	New Zealand	-	-	-
18	*Pseudomonas syringae* pv. *antirrhini*[T]	NCPPB 1817	United Kingdom	NT[d]	NT	-
19	*Pseudomonas syringae* pv. *berberidis*[T]	NCPPB 2724	New Zealand	NT	NT	-
20	*Pseudomonas syringae* pv. *persicae*[T]	NCPPB 2761	France	NT	NT	-
21	*Pseudomonas syringae* pv. *lachrymans*[T]	NCPPB 2916	Zimbabwe	NT	NT	-
22	*Pseudomonas syringae* pv. *maculicola*[T]	LMG 5071	New Zealand	NT	NT	-
23	*Pseudomonas syringae* pv. *tomato*[T]	LMG 5093	United Kingdom	NT	NT	-
24	*Burckholderia gladioli* pv. *gladioli*[T]	LMG 2216	United States	NT	NT	-
25	*Xanthomonas campestris* pv. *campestris*[T]	LMG 568	United Kingdom	NT	NT	-
26	*Xanthomonas axonopodis* pv. *citri*	KACC 10443	Republic of Korea	NT	NT	-
27	*Xanthomonas axonopodis* pv. *vesicatoria*	LMG 905	Unknown	NT	NT	-
28	*Xanthomonas campestris* pv. *vesicatoria*	LMG 921	United States	NT	NT	-
29	*Ralstonia solanasearum*[T]	LMG 2299	United States	NT	NT	-
30	*Erwinia persicina*[T]	LMG 11254	Japan	NT	NT	-
31	*Pectobacterium carotovorum* subsp. *carotovorum*[T]	LMG 2404	Denmark	NT	NT	-
32	*Pectobacterium atrosepticum*[T]	KACC 10477	United Kingdom	NT	NT	-
33	*Pectobacterium wasabiae*[T]	LMG 8444	Japan	NT	NT	-
34	*Escherichia coli*[T]	LMG 2092	Denmark	NT	NT	-

KACC, Korean Agricultural Culture Collection, Republic of Korea (http://www.genebank.go.kr/); LMG, The Belgian Co-ordinated Collections of Microorganisms (BCCM[TM]), Belgium; NCPPB, National Collection of Plant Pathogenic Bacteria.

[a] T: type strain.

[b] Ferredoxin reductase gene of *C. michiganensis* subsp. *michiganensis* NCPPB 382. Positions Cmm141F/R correspond to GenBank accession no. NC_006834, gi|58579623: 124816-125631.

[c] +, detected; -, not detected.

[d] NT, not tested.

Table 2. Sequences of the primers and probes used in this study

Primer	Sequences (5'-3')	Annealing temp. (°C)	Amplicon size (bp)	Gene or Position	Reference
CMM-5	GCGAATAAGCCCATATCAA	55	609	*pat*-1	Dreier *et al.* (1995)
CMM-6	CGTCAGGAGGTCGCTAATA				
PSA-4	TCATTGGTCAATTCTGTCTCCC	63	290	16S-23S rRNA	Pastrik and Rainey (1999)
PSA-R	TACTGAGATGTTTCACTTCCCC				
Cmm141F	CAGGCGTCCGTCGGTGAGGTGGTC	63	141	140237-140377[a]	This study
Cmm141R	GCGGGAGAGCGGTGCGGGAATG				

[a] Positions correspond to GenBank accession No. AM711867.1, gi|148271178:139748-140950.

used according to the Handbook of Microbiological Media (Atlas, 2004): *Clavibacter* and *Xanthomonas* species on YGC medium (2.0% D-(+)-glucose, 2.0% $CaCO_3$, 1.0% yeast extract, and 1.5% agar) for 2–3 days at 28°C, other microbes on LB agar (Difco, USA) at 28°C for 24 h and *Escherichia coli* on Nutrient agar (Difco) at 37°C for 24 h.

DNA extraction

All of the bacterial strains were cultured on optimal media and harvested with a scraper for genomic DNA extraction, which was prepared using a DNeasy Tissue kit (QIAGEN, Germany) according to the manufacturer's protocols. The genomic DNA concentrations were measured using a NanoDrop® ND-1000 Spectrophotometer (NanoDrop Technologies, USA), and genomic DNA exhibiting a spectrophotometric A260/A280 ratio between 1.8–2.1 and A260/A230 ratio of 1.8–2.2 were used. The genomic DNA of all of the bacterial strains was diluted in TE buffer (pH 8.0) to 25 ng/μl.

Primer design and conventional PCR

A primer pair was designed for the ferredoxin reductase gene of *C. michiganensis* subsp. *michiganensis* NCPPB 382 (GenBank Accession No. AM711867.1, gi|148271178:139748-140950), with a predicted PCR product of 141 bp (Table 2). All amplifications were performed with these primers (0.5 μM final concentration) and GoTaq® DNA polymerase (1.25 μ final concentration; Promega, USA) according to the manufacturer's instructions and approximately 50 ng genomic DNA. The amplifications were performed using a PTC-225 thermocycler (MJ Research, USA) with the following cycling conditions: initial denaturation of 5 min at 95°C; 35 cycles of 1 min at 95°C, 30 sec at 63°C, and 1 min at 72°C; and a final extension of 7 min at 72°C. After the PCR reaction, each amplified PCR product was electrophoresed through a 1.5% agarose gel, stained with ethidium bromide, and visualized on an UV transilluminator and imaged using a VersaDoc 1,000 gel imaging system (Bio-Rad Laboratories, Inc., USA).

For the amplification of the plasmid-borne pathogenicity gene (*pat*-1) and 16S–23S rRNA intergenic spacer region, the primers and reaction conditions were followed by Dreier *et al.* (1995), Pastrik and Rainey (1999), and Palacio-Bielsa *et al.* (2009). The nucleotide sequences of the *pat*-1 and 16S-23S rRNA are presented in Table 2 (Dreier *et al.*, 1995; Pastrik and Rainey, 1999; Palacio-Bielsa *et al.*, 2009).

Specificity and sensitivity of the SYBR Green real-time PCR assay

The SYBR Green real-time PCR assay was performed in a 20 μl reaction. All of the amplifications were performed with the primers describe above (0.5 μM final concentration) and iQ™ SYBR® Green Supermix (Bio-Rad Laboratories, Inc.), according to the manufacturer's instructions, and approximately 5 ng of purified DNA was used from each sample. The real-time PCR amplifications were performed using a CFX96 real-time PCR system (Bio-Rad Laboratories, Inc.) and the following cycling conditions: initial denaturation of 3 min at 95°C and 45 cycles of 10 sec at 95°C and 20 sec at 63°C and a melting curve 65°C to 95°C, with an increment of 0.5°C. Determination of the cycle threshold (*Ct*) and the data analysis were set automatically by the CFX Manager™ Software system (Version 1.6; Bio-Rad Laboratories, Inc.). The copy number of the cloned DNA was calculated with the following equation (Whelan *et al.*, 2003): copies/μl = [6.022× 10^{23} (copy/mol)×amount (g)] / [length (bp)×660 (g/mol/bp)].

Fig. 1. Sampling area of the tomato seedlings (*Lycopersicon esculentum* cv. Juok). The assays were performed using the root-cutting method with *C. michiganensis* subsp. *michiganensis* LMG7333.

Detection of the pathogen by quantitative direct PCR

For the pathogenicity tests, tomato (*Lycopersicon esculentum.* cv. Juok) seedlings were grown in pots (10 cm diameter), with one plant/pot, for 4 to 5 weeks in a shaded greenhouse at 20 to 25°C. *C. michiganensis* subsp. *michiganensis* LMG7333 was grown on YGC medium, and bacterial cells in suspension was adjusted to OD_{600}=0.11. An inoculation assay was conducted with the bacterial cells suspension according to the cut-root method (Ozaki and Kimura, 1989). Samples were collected from four different areas at 12 days post inoculation (dpi) (Fig. 1). For the seed test experiment, five naturally infected seeds (tomato and red pepper) were provided by National Plant Quarantine Service, Republic of Korea were used in each PCR reaction. Each sample was dipped in 500 μl sterile distilled water for 30 min, 2 μl of the water treated with each sample was used in the SYBR Green real-time and conventional PCR assays as described above.

Results

Sequence specificity of the designed primers

The specific primer pair was designed based on the sequences of the ferredoxin reductase gene of *C. michiganensis* subsp. *michiganensis* NCPPB 382 (No. AM711867.1, gi|148271178: 139748-140950). The specificity of the primer pair was tested *in silico* by similarity search against the NCBI-BLAST sequence database (http://www.ncbi.nlm.nih.gov/).

The results showed some significant matches with previously determined sequences (BLASTN ver. 2.2.25+), and the BLASTN searches showed similarity to the ferredoxin reductase sequences [identity=67%, score=120 bits (132), and expected=3e-23] from *Streptomyces scabiei* 87.22. BLAST searches with the predicted protein sequence (BLASTX) revealed similarity to the ferredoxin reductase sequence [identity=49%, score=343 bits (880), and expected=3e-92] from *Streptomyces* sp. SA3_actG. However, the region to be amplified with the designed primer pair revealed no significant match in either the BLASTN or BLASTX searches.

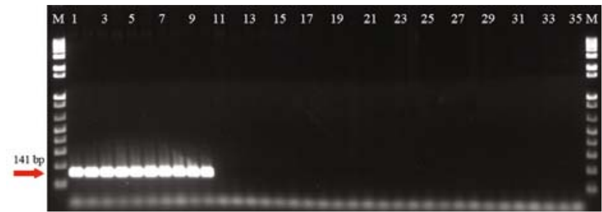

Fig. 2. Specific PCR amplification of a ferredoxin reductase gene fragment from *C. michiganensis* subsp. *michiganensis* using the Cmm141F/R primer set. Lanes: M, standard size marker (1 kb DNA plus ladder; Gibco BRL); 1–34, are described in Table 1; lane 35, distilled water.

Specificity of the PCR assay

The Cmm141F/R primer pair was tested to assess check the specificity of the primers for both the conventional and SYBR Green real-time PCR analyses (Table 1). As expected, a 141 bp DNA fragment was amplified by conventional PCR (Fig. 2). In contrast, the amplification of a DNA fragment was not detected in the other *Clavibacter* subspecies strains or reference microorganisms; only the assays with *C. michiganensis* subsp. *michiganensis* yielded a single amplified DNA fragment with a fluorescence intensity.

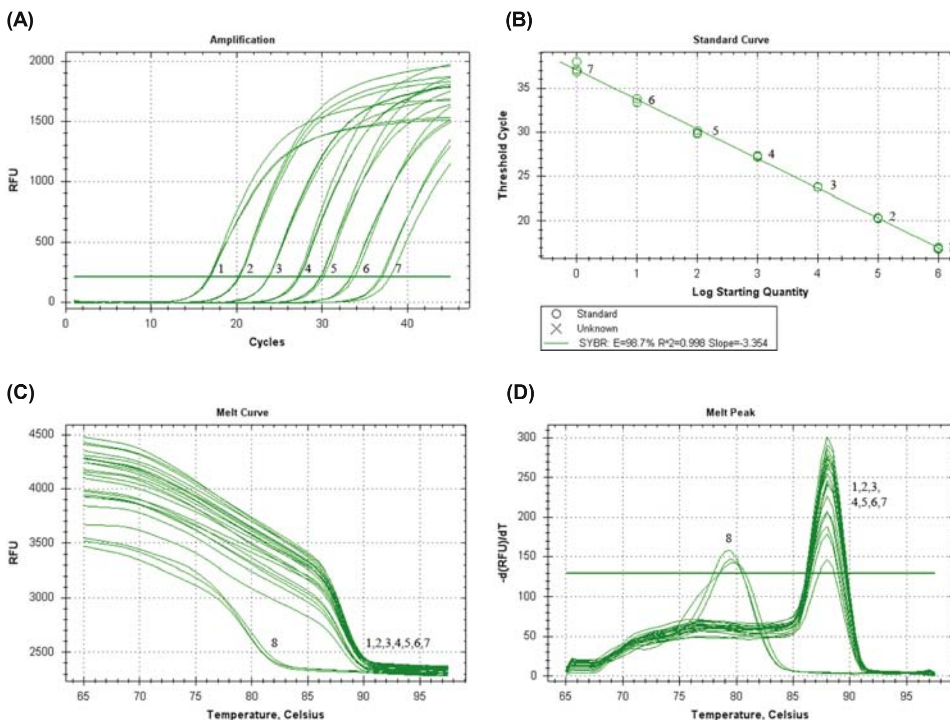

Fig. 3. Specificity, melting peak and standard curve of the Cmm141F/R primer set using SYBR Green real-time PCR. (A) Fluorescence intensity as a function of the concentration of the template. For each assay, a series of 10-fold dilutions of cloned DNA (range, 1.47×10^9 to 1.47×10^3 copies/μl) were used as the template for PCR (1–7, sample dilutions). (B) Standard curve derived from the amplification plot. (C) Melting-curve analysis (1–7, sample dilutions; 8, no template control). (D) Melting-peak analysis (1–7, sample dilutions; 8, no template control). The negative first derivative of the relative fluorescence units [–d(RFU)/dT] is plotted as a function of the temperature. Amplified product, 88.5°C. A high peak indicates an amplified product; the low peak is the no-template control .

Table 3. **Mean *Ct* end-point fluorescence of 10-fold serial dilutions of *C. michiganensis* subsp. *michiganensis* cloned DNA, genomic DNA and cell suspension as determined by the SYBR Green real-time PCR assay**

Cloned DNA		Genomic DNA		Cell suspension	
Plasmid copies/µl	*Ct* values	Weight/µl	*Ct* values	Cell density[a]	*Ct* values
1.47×10^{9}	16.90±0.11	5 ng	15.51±0.21	0.11×10^{0}	20.10±0.31
1.47×10^{8}	20.31±0.07	500 pg	18.37±0.15	0.11×10^{-1}	21.40±0.10
1.47×10^{7}	23.79±0.03	50 pg	21.60±0.10	0.11×10^{-2}	25.56±0.07
1.47×10^{6}	27.27±0.12	5 pg	25.24±0.15	0.11×10^{-3}	29.49±0.03
1.47×10^{5}	29.98±0.20	500 fg	28.60±0.29	0.11×10^{-4}	32.86±0.27
1.47×10^{4}	33.53±0.28	50 fg	32.21±0.09	0.11×10^{-5}	35.95±0.24
1.47×10^{3}	37.32±0.69	5 fg	35.22±0.18	0.11×10^{-6}	38.56±1.44

[a] OD 600nm unit of cells.

Sensitivity and quantitative performance

We used an SYBR Green real-time PCR analysis of *C. michiganensis* subsp. *michiganensis* to generate a standard curve by plotting the mean *Ct* (n=3) versus the logarithmic concentration of the cloned DNA, genomic DNA and density of the cell suspension (range, 1.47×10^{9} to 1.47×10^{3} copies/µl, 5×10^{0} to 5×10^{-6} ng/µl and 0.11×10^{0} to 0.11×10^{-6} OD at 600 nm units of cells, respectively) (Fig. 3A and Table 3). The assay exhibited a good linear response (R^2=0.998). The detection limit of the reaction was one cloned copy of the ferredoxin reductase gene of *C. michiganensis* subsp. *michiganensis*. Standard regression analysis of the linear part of the slope gave a coefficient of -3.354, which yielded a PCR efficiency of 98.7% (Fig. 3B). The melting curve derived from the amplification plot is shown in Fig. 3C, and the analysis of the melting temperature and melting peaks of *C. michiganensis* subsp. *michiganensis* by SYBR Green real-time PCR revealed a reproducible Tm of 88°C and specific peaks (Fig. 3D).

Detection of the pathogen by quantitative direct PCR in naturally infected tomato samples

The primer pair of Cmm141F/R also detected the pathogen by SYBR Green real-time PCR in naturally infected tomato samples. Amplified products derived from naturally infected tomato and red pepper samples showed fluorescence using the SYBR Green real-time PCR assay, whereas no signals were amplified from the healthy tomato and red pepper samples (Fig. 4). The infected tomato seeding and naturally infected seed samples showed *Ct* value ranging from 22 to 25. The horizontal green line represents the threshold, which was set automatically by the instrument.

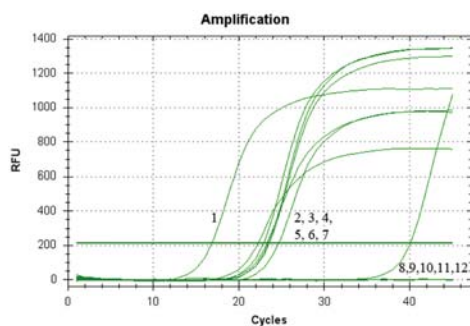

Fig. 4. **Specific detection of *C. michiganensis* subsp. *michiganensis* by SYBR Green real-time PCR in infected tomato seedling and naturally infected seeds (tomato and red pepper) using the Cmm141F/R primer set.** Fluorescence intensity corresponding to ferredoxin reductase gene sequences from *C. m.* subsp. *michiganensis* amplified using Cmm141F/R. 1, *C. michiganensis* subsp. *michiganensis* LMG7333 genomic DNA; 2–5, inoculated tomato seedlings; 6, naturally infected tomato seeds; 7, naturally infected red pepper seeds; 8–9, healthy tomato seedlings; 10-11, healthy seeds (tomato and red pepper), respectively; 12, no template control (distilled water).

Discussion

The objective of this research was to develop a highly sensitive and specific method that could aid in the detection of *C. michiganensis* subsp. *michiganensis* in infected tomato seed lots. Much research has been performed to develop sensitive and specific technologies to improve seed-borne pathogen detection and ensure successful control measures (Rademaker and Janse, 1994; Dreier *et al.*, 1995; Leon *et al.*, 2006). However, each of these techniques has advantages and disadvantages related to their lack of sensitivity and/or specificity, cost or time factors, and the requirement of chambers under controlled conditions for bioassays. In particular, as the main source for the transmission of *C. michiganensis* subsp. *michiganensis* is contaminated seeds and transplants, the control of this plant disease requires reliable and sensitive methods for detecting the pathogen in these tissues. Immunofluorescence and ELISA are hampered by the fact that the available antibodies lack specificity to allow differentiation at the subspecies level (Franken *et al.*, 1993).

A currently available primer set was reported to have 95% homology with the ITS of other subspecies. Although the ITS was amplified only from strains of *C. michiganensis* subsp. *spedonicus*, it differed at only two or three bases with respect to the analogous regions in four closely related subspecies, thus requiring more rigid control of the annealing temperature to maintain primer specificity (Baer *et al.*, 2001). In addition, the *pat*-1 and 16S–23S rRNA primer sets failed to generate the specific amplicon listed in Table 1 (Dreier *et al.*, 1995; Pastrik and Rainey, 1999).

In the present study, we report a highly validated, specific, sensitive, and reliable quantitative direct PCR assay for the quantitative detection of *C. michiganensis* subsp. *michiganensis*. The assay shows excellent quantification characteristics, attaining accurate detection.

A bioinformatics method was used to search for specific

target sequences for the PCR detection of bacterial pathogens and included genomic comparisons of candidate gene with other bacterial DNA genomic sequences via BLAST (Zhu *et al*., 2009).

Using a sequence alignment search, we found a specific region of the ferredoxin reductase gene of *C. michiganensis* subsp. *michiganensis* and *C. michiganensis* subsp. *spedonicus* using BLAST and e-PCR analysis (Park *et al*., 2006, 2009). It has been shown that ferredoxin reductase is a ubiquitous flavoenzyme, containing noncovalently bound FAD as a prosthetic group. Ferredoxin reductase plays a role in delivering NADPH or low potential one-electron donors such as ferredoxin and flavodoxin, during redox-based metabolism in plastids, mitochondria and bacteria. However, flavoprotein pyridine nucleotide cytochrome reductases (FPNCRs) show no sequence similarity to NADPH:adrenodoxin reductases or to bacterial ferredoxin:NAD reductases and their homologs, despite their functional similarities (http://www.ebi.ac.uk/interpro/IEntry?ac=IPR015701).

In general, the detection limits reported for the other techniques of *C. michiganensis* subsp. *michiganensis* detection are lower: 10^4 cells/ml were necessary for a positive ELISA reaction with pure cultures (Gitaitis *et al*., 1992) and the PCR diagnosis protocol using *C. michiganensis* subsp. *michiganensis* primers developed by Dreier *et al*. (1995) detected 10^2 cells/ml. To our knowledge, there are not many other techniques that have been developed in recent years for the detection of *C. michiganensis* subsp. *michiganensis*, and the most sensitive procedure remains the isolation of the microorganism on semiselective media such as SCM, which detects a single contaminated seed containing 50 CFU in samples of 10,000 seeds (Fatmi and Schaad, 2002).

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