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RESEARCH ARTICLE

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A multiplex PCR method for detection of *Clavibacter michiganensis* subsp. *michiganensis* with co-amplification of its host DNA

Abstract A multiplex PCR assay system was developed for the detection of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), which combined two tests in one reaction mixture. Cmm-specific primers PSA-4/PSA-R and *Solanum lycopersicum*–specific primers NS-7-F/NS-8-R (internal PCR control primer) were combined in one PCR reaction mixture with Cmm and plant DNA as template. The primer sets could amplify the target product successfully. Different combinations and concentrations of primers and annealing temperatures were tested, respectively. The detection level of the optimized multiplex PCR assay was up to 5×10^2 cfu·mL^{-1}. To verify the applicability of this system, it was employed to detect Cmm in tomato seeds and plantlet samples. Seeds mixed with Cmm and diseased plantlets were detected successfully. The multiplex PCR system will avoid false-negative results and provide a reliable method for the detection of Cmm.

Keywords *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), molecular detection, multiplex PCR, internal PCR control, false negative

1 Introduction

Bacterial canker, caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), is a harmful bacteriosis on tomato crops, causing great economic losses in commercial tomato production areas. Until now, tomato bacterial canker has been observed in Beijing, Heilongjiang, Liaoning, Inner Mongolia, Xinjiang, Hebei, Shanxi, Shandong, Shanghai, Hainan, and other areas throughout China, and has brought about losses at different levels (Luo et al., 2004). Infected seeds are considered the primary inoculum source (Gitaitis et al., 1992). The sporadic but devastating nature of this disease, the capability of Cmm to survive several months on plant debris and seeds under natural conditions, and the absence of effective measures on infected crops, make bacterial canker a potential threat for all tomato-producing areas. The most important strategy for controlling bacterial canker is to use pathogen-free seeds and seedlings (Janse and Wenneker, 2002). Therefore, early detection may ensure successful control of this canker.

Current test procedures belong to serological techniques such as ELISA and immunofluorescence (IF)-assay. However, they are not always reliable due to their cross-reactions with other bacteria and limited sensitivity (Dreier et al., 1995). Plating seed extracts on semi-selective media (EPPO/CABI, 2005) or bioassay (inoculating host plants with suspicious contaminated extracts) was time-consuming and easily affected by circumstances. PCR assays are considered rapid and sensitive. But PCR assays may be hindered by some factors, including the absence of the pathogen and failed DNA isolation or by inhibition factors in the PCR reaction (Sousa Santos et al., 1997). In the two latter cases, false-negative results were obtained. *Taq* DNA polymerase is sensitive to inhibition by factors present in biological samples, and a number of chemicals used in the DNA extraction procedure were found to interfere with DNA amplification. Therefore, the exclusion of false-negative results is essential for the use of PCR-based tests as screening assays.

Multiplex PCR combines several primer sets in the same PCR assay, providing a means of reliable pathogen detection in routine testing. Our study described the development of a multiplex PCR for the detection of Cmm, in which co-amplification of host DNA provides a novel

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internal PCR control, thus improving the reliability of the PCR assay for routine screening of tomatoes for phytopathogenic bacteria. This method has not been applied to Cmm, although it has been used in the detection of *Clavibacter michiganensis* subsp. *Sepedonicus* (Pastrik, 2000).

The main objective was to develop a reliable screening PCR assay for the detection of the pathogen Cmm. Multiplex PCR reaction conditions were optimized (Elnifro et al., 2000), and the optimized detection system was employed to detect Cmm in tomato seeds and plantlet samples in order to get a reliable detection system.

2 Materials and methods

2.1 Materials

The Cmm tested in this study was obtained from China Agricultural University and grown on Yeast Peptone Glucose Agar (EPPO/CABI, 2005) at 26°C. Tomato seeds were purchased at Baoding market.

2.2 Reagent

Taq DNA polymerase, pMD-19T Vector, and *Escherichia coli* JM109 were bought from Takara Biotechnology Company. EZ spin column DNA gel extraction kit and DNA marker were the products of Shanghai Sangon Biological Engineering Technology and Services Company.

2.3 DNA extraction

2.3.1 DNA extraction of Cmm

The bacteria were suspended in sterile distilled water in a microvial. Closed vials were heated at 95°C for 15 min. Microvials with heated suspensions were transferred into ice and after cooling, centrifuged the supernatant was used as template (EPPO/CABI, 2005).

2.3.2 DNA extraction from inoculated tomato leaves

Tomato leaves mixed with lysis buffer (100 mmol·L^{-1} NaCl; 10 mmol·L^{-1} Tris-HCl, pH 8.0; 1 mmol·L^{-1} EDTA, pH 8.0) were triturated by electric drill, placed on a heating block at 95°C for 10 min, and cooled on ice for 5 min. Lysozyme was added, and the sample was incubated for 30 min at 37°C. After the addition of proteinase K, the mixture was incubated for 30 min at 55°C. Then CTAB was added and the mixture was incubated for 30 min at 65°C. Phenol/chloroform/isoamyl alcohol (25∶24∶1) was added to separate DNA. The mixture was centrifuged, the aqueous phase was transferred to a new tube, and DNA was precipitated with 96% ethanol. The resulting pellet was dissolved in sterile water.

2.3.3 DNA extraction from Cmm-mixed seeds

Seeds mixed with Cmm were triturated in mortar accompanied with liquefiable nitrogen. The triturated sample was transferred into a centrifuged tube, and the following procedure was as described in 2.3.2.

2.4 Primers and PCR amplification

For the specific amplification of Cmm, the pathogen-specific primer set PSA-1/PSA-R was used, which was based on the intergenic spacer region (16S-23S rRNA) of Cmm (Pastrik and Rainey, 1999) with a predicted PCR product of 271 bp. For the amplification of the internal PCR control, the primer set NS-7-F/NS-8-R (White et al., 1990) was employed. These primers, which are based on conserved nucleotide sequences from 17S rRNA genes from *Solanum lycopersicum*, amplify a DNA fragment of 376 bp.

PCR was performed in a PTC 200 thermocycler (Mastercycler gradient, eppendorf). The PCR reaction mixture (25 μL) contained 1X Reaction Buffer; 100 μmol·L^{-1} of each dNTP, 0.4 μmol·L^{-1} each of primers PSA-1/PSA-R and 0.07 μmol·L^{-1} each of primers NS-7-F/NS-8-R, 1.25U *Taq* DNA polymerase, and 2 μL of the DNA solution. The PCR conditions were: initial denaturizing at 95°C for 3 min, followed by 30 reaction cycles of 95°C for 1 min, 63°C for 1 min, and 72°C for 1 min, then keeping at 72°C for 5 min. 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 ethidium bromide.

2.5 Optimization of the multiplex PCR

2.5.1 Proportion of primers

The optimization experiments were performed with DNA extracts from inoculated tomato. The ratio of the primer sets PSA-1/PSA-R and NS-7-F/NS-8-R were tested in order to achieve high sensitivity of detection. The concentration of each primer PSA-1/PSA-R added was the same and so as NS-7-F/NS-8-R. The ratios of primers varied while the total amount was fixed. The concentrations added are shown in Table 1.

2.5.2 Concentration of primers

Different concentrations of primers were tested on the basis of fixed proportion. The amounts of primers added are shown in Table 2.

Table 1 Concentration and ratio of the two specific primers used in the reaction system

item	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
PSA/(μmol·L^{-1})	0.75	0.72	0.70	0.67	0.66	0.64	0.6	0.53	0.40	0.34	0.2	0.13	0.10	0.08	0.05
NS/(μmol·L^{-1})	0.05	0.08	0.10	0.11	0.13	0.16	0.2	0.27	0.40	0.67	0.6	0.67	0.70	0.72	0.75
ratio	15.00	9.00	7.00	6.00	5.00	4.00	3.00	2.00	1.00	0.50	0.30	0.20	0.15	0.10	0.50

Table 2 Different concentrations of the primers used in the reaction system

item	1	2	3	4	5	6	7	8	9
PSA/(μmol·L^{-1})	1.38	1.03	0.83	0.69	0.55	0.40	0.28	0.15	0.07
NS/(μmol·L^{-1})	0.23	0.15	0.14	0.14	0.09	0.07	0.05	0.02	0.01

2.5.3 Optimization of annealing temperature

Different annealing temperatures (51.0°C, 51.3°C, 52.5°C, 54.2°C, 56.5°C, 59.1°C, 61.8°C, 64.5°C, 67.1°C, 69.1°C, 70.6°C, and 71.5°C) were tested in order to get a good amplification result.

2.6 Cloning of the PCR product and sequencing

The PCR products were purified and linked with pMD19-T vector, transforming the plasmid into the *E. coli* competent cell JM109. The positive clone was sent to Shanghai Sangon Biological Engineering Technology and Services Company for sequencing.

2.7 Detection of tomato and its seeds by the optimized multiplex system

Tomato seedlings with two to three true leaves were inoculated with Cmm by needle penetrating. Leaf samples were taken 2, 4, 6, 8, 10, 15 and 20 days after inoculation. DNA was extracted from these leaves and used as template with healthy plant DNA as control. The disease symptom was recorded. Disease indexes (Luo et al., 2005) were as follows:

0 level: leaves or cotyledon did not scorch or caudexes did not show putrescence;

1 level: leaves or cotyledon scorched slightly;

2 level: leaves or cotyledon turned yellow or withered moderately;

3 level: leaves or cotyledon withered or caudexes showed putrescence seriously;

4 level: plants died.

Tomato seeds were mixed with serial dilution of cell suspension (0, 5×10^2 cfu·mL^{-1}, 5×10^3 cfu·mL^{-1}, 5×10^4 cfu·mL^{-1}, 5×10^5 cfu·mL^{-1}, 5×10^6 cfu·mL^{-1}) to mimic natural contaminated seeds. DNA was extracted from 30 grains of seeds at different levels.

3 Results

3.1 Uniplex and mutiplex PCR amplification

DNA extracted from the inoculated tomato was used as template. DNA fragments of 271 bp (Cmm-specific) were amplified with primer set PSA-4/PSA-R, DNA fragments of 376 bp (plant-specific) were obtained with primer set NS-7-F/NS-8-R, and two discrete DNA fragments at the size of 271 bp (Cmm-specific) and 377 bp (plant-specific) were generated by multiplex PCR. These results were consistent with what was expected.

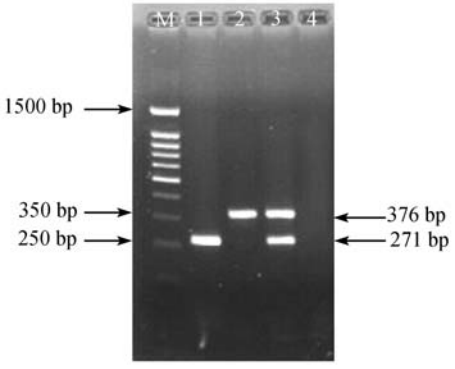

Fig. 1 Amplification results of uniplex PCR and multiplex PCR Note: M represents DNA size marker 100 + 50 bp ladder. Lanes 1, 2, 3, and 4 represent amplification by primer PSA-4/PSA-R, amplification by primer NS-7-F/NS-8-R, amplification by primer PSA-4/PSA-R and NS-7-F/NS-8-R, and negative control, respectively.

The PCR fragments were cloned and subjected to sequencing. Homology analysis revealed that the sequences obtained in the study were 100% homologous with corresponding sequences in GenBank.

3.2 Effect of different proportions and concentrations of primers to multiplex PCR

The relative proportions and concentrations of the primer sets PSA-4/PSA-R and NS-7-F/NS-8-R were critical for the sensitivity of the PCR assay. As shown in Fig. 2, 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 Cmm. Several combinations of primer proportions were

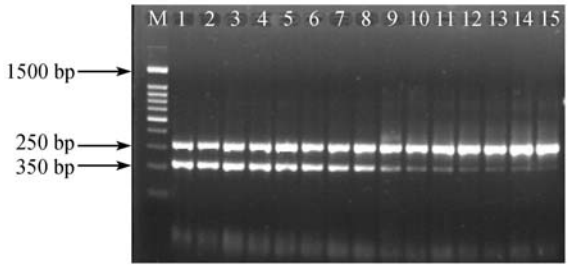

Fig. 2 The effect of different ratios of two pairs of primers
Note: M represents marker; Lanes 1–15 correspond to the series in Table 1.

tested, and the highest sensitivity was achieved with 0.7 μmol·L^{-1} of primers PSA-4/PSA-R and 0.1 μmol·L^{-1} of primers NS-7-F/NS-8-R. Different concentrations of primers were tested on the basis of the proportion. The results showed that good amplification results could be obtained when the concentration of primer PSA-4/PSA-R and primer NS-7-F/NS-8-R were increased over 0.4 and 0.07 μmol·L^{-1}, respectively; however, the further increase in the primer concentration would produce more dimmer of primers (Fig. 3). Consequently, 0.4 μmol·L^{-1} of primer PSA-4/PSA-R, and 0.07 μmol·L^{-1} of primer NS-7-F/NS-8-R were chosen as the best concentrations, respectively.

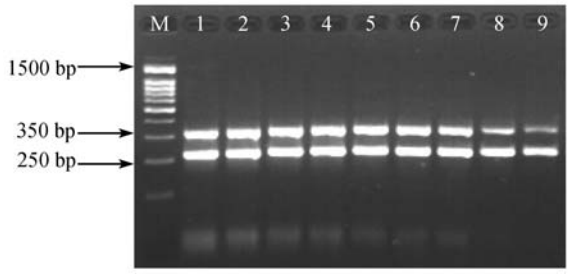

Fig. 3 The effect of different concentrations of two sets of the primers
Note: M represents marker; Lanes 1–15 correspond to the series in Table 2.

3.3 Optimization of annealing temperature

Non-specific amplification would appear when the annealing temperature was below 56.5°C, and the amplified fragments were almost the same when the annealing temperature was above 59.1°C (Fig. 4). In order to assure the specific amplification, 63°C was chosen according to Pastrik and Rainey (Pastrik and Rainey, 1999).

3.4 Sensitivity test

Using the optimized multiplex PCR protocol, a sensitivity test was carried out using healthy tomato DNA mixed with serial dilution of cell suspension. Detection sensitivity was

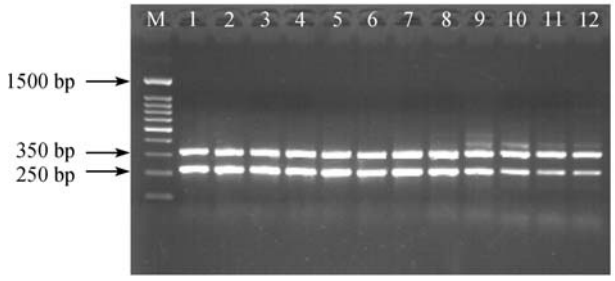

Fig. 4 The effect of different annealing temperatures on amplification
Note: M represents marker; Lanes 1–12 represent 71.5°C, 70.6°C, 69.1°C, 67.1°C, 64.5°C, 61.8°C, 59.1°C, 56.5°C, 54.2°C, 52.5°C, 51.3°C, and 51.0°C, respectively.

compared with the primer set PSA-4/PSA-R in uniplex PCR.

It was possible to detect artificially added Cmm of 5×10^2 cfu·mL^{-1} in PCR reaction mixture. This was equivalent to the detection sensitivity of the uniplex PCR using primer set PSA-1/PSA-R (Fig. 5).

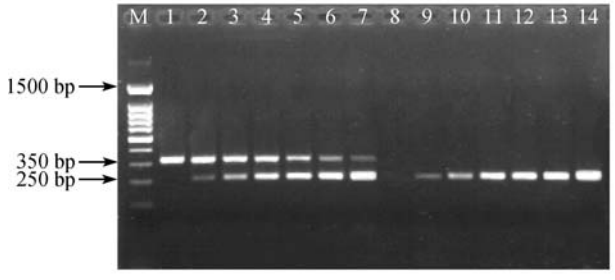

Fig. 5 Sensitivity of the multiplex PCR and the uniplex PCR for Cmm
Note: M represents marker; Lanes 1–7 represent the sensitivity of multiplex PCR with healthy tomato DNA mixed with dilutions of Cmm cells ranging from 50 to 5×10^7 cfu·mL^{-1}; Lanes 8–14 represent the sensitivity of uniplex PCR with healthy tomato DNA mixed with dilutions of Cmm cells ranging from 50 to 5×10^7 cfu·mL^{-1}.

3.5 Detection of tomato and seeds by the optimized multiplex system

Tomato showed slightly scorched appearance and the leaflets along one side became flaccid 10 days after inoculation. Leaves wilted and shriveled 15 days after inoculation. Symptoms including wilted leaves, stem necrosis, and death appeared among plantlets 20 days after inoculation. Disease index was appraised at the 0, 0, 0, 1 and 2 levels on the sampling time 2, 4, 6, 8, 10 and 15 days after inoculation and at the 3 and 4 levels 20 days after inoculation corresponding to different symptoms. DNA from different stage samples was used in multiplex PCR. Results are shown in Fig. 6. Cmm could be detected 6 days

after inoculation when the plants did not show any symptom. The amount of Cmm-specific fragments became larger gradually 6 days after inoculation but remained stable 15 days after inoculation, which indicated the growth of Cmm in plants became stable.

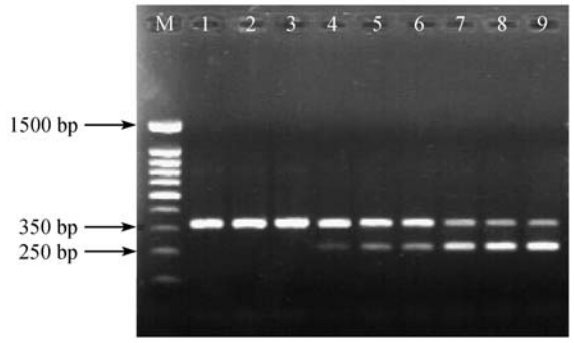

Fig. 6 Direct detection of inoculated tomato leaves with multiplex PCR
Note: M represents marker; Lanes 1–9 represent healthy plants, 2 days after inoculation (0 level), 4 days after inoculation (0 level), 6 days after inoculation (0 level), 8 days after inoculation (0 level), 10 days after inoculation (1 level), 15 days after inoculation (2 level), 20 days after inoculation (3 level), and 20 days after inoculation (4 level), respectively.

The results of seed detection are shown in Fig. 7. Seeds mixed with 5×10^4 cfu·mL^{-1} could be detected, indicating that multiplex PCR could also be used in seed detection.

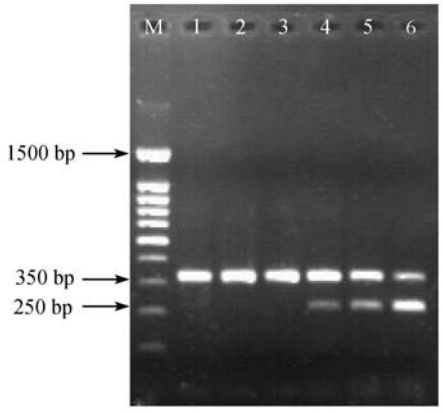

Fig. 7 Detection of tomato seeds artificially mixed with Cmm
Note: M represents marker; Lanes 1–6 represent Cmm-free seeds, seeds soaked with 5×10^2 cfu·mL^{-1}, seeds soaked with 5×10^3 cfu·mL^{-1}, seeds soaked with 5×10^4 cfu·mL^{-1}, seeds soaked with 5×10^5 cfu·mL^{-1}, and seeds soaked with 5×10^6 cfu·mL^{-1}, respectively.

4 Discussion

Multiplex PCR amplification (Chamberlain et al., 1988) provides a way 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. The application of multiplex PCR in pathogen detection has two major purposes: first, primers are targets to one specific gene of each pathogen, so several kinds of pathogen can be detected in one PCR reaction; second, primers are targets to several genes of one pathogen in order to avoid false-positive detection results. The aim of the multiplex PCR assay in this research was different. The PCR assay was combined with two different tests in one reaction mixture, including a highly specific and sensitive detection of the pathogen and an indicator test for successful amplification (internal PCR control), which monitored potentially false-negative PCR results derived from failure reaction of the PCR. DNA extracted from plants or seeds could be used as template for PCR assay without pathogen culture. The assay could indicate the success of DNA isolation and avoid false-negative results, which could improve the reliability of detection. Amplification of host plant DNA as an internal control has been used in the detection of *Clavibacter michiganensis* subsp. *sepedonicus* (Pastrik, 2000), but not in Cmm.

Multiplex PCR and the simultaneous amplification of more than one DNA region of interest in one reaction mixture required specific primers to assure the accuracy of detection. Primers usually used for bacterial detection were targeted to 16S rRNA, *ITS*, or pathogenicity-related genes. 16S rRNA was very conservative among *Clavibacter michiganensis* subsp., which could not differentiate subspecies. Both plasmids of Cmm harbored genes that were involved in pathogenicity. Primers and probes were designed according to the *pat-1* gene related with pathogenicity (Dreier et al., 1995), which could differentiate pathogenic and unpathogenic bacteria. Bacteria without plasmids could also survive in plants with no disease symptoms (Burger et al., 2005). The plasmids of Cmm comprised transfer factors which would allow regaining the plasmids from an appropriate donor after they were lost. In this way, PCR assay based on plasmids would underestimate the potential pathogenicity. The ITS region, a short sequence between 16S and 23S rRNA, varied among subspecies and was relatively conservative. Primers designed among *ITS* were widely used in the detection, which could discriminate subspecies. Primers designed by Pastrik and Rainey (1999) with amplification of 271-bp fragments were specific and widely used (EPPO/CABI, 2005; Fu et al., 2005). The inner control was targeted to the 17S rRNA gene region with amplification of 376-bp fragments. The two sets of primers were specific and could avoid non-specific amplification, the annealing temperatures were alike and could react in one mixture, and the length of amplification fragments was different and could be distinguished easily. All the factors confirmed a successful multiplex PCR.

The optimization of factors, especially the concentrations of primers, was essential for a successful multiplex

PCR assay (Zhang et al., 2003). Higher concentrations of primer set NS-7-F/NS-8-R could result in an increase in the amplification of plant-specific DNA fragment, but decreased the detection sensitivity for Cmm. The sensitivity of detection was 5×10^2 cfu·mL^{-1} (10–15 cfu per PCR reaction mixture), which was equivalent to the detection sensitivity of the uniplex PCR (Pastrik and Rainey, 1999).

The optimized assay was employed to detect Cmm in tomato seeds and plantlet samples. Cmm could be detected from leaves 6 days after inoculation, which was consistent with Zhao (2007). Different sampling and detection ways might lead to different results. Cmm could be detected by Burokienė (Burokienė, 2006) 3 days after inoculation. Different sampling sites bore different amounts of bacteria while the bacteria were growing in inner plants. The sampling sites chosen by Burokienė were 8 cm away from the inoculation site and the bacteria were cultured on the medium before using as template (Bio-PCR). Samples used in this paper were obtained from leaves, and DNA extracted from plants was used as template directly without bacteria culture. This method was fast and could avoid problems brought about by improper culturing conditions.

Seeds soaked by 5×10^4 cfu·mL^{-1} bacteria suspension could be detected but the lower concentration could not, which was related to the amount of seeds used for DNA extraction and lost during extraction.

In conclusion, the multiplex PCR protocol is sensitive and reliable for the detection of Cmm. It provides a useful tool to monitor the occurrence of Cmm in seeds and plants.

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