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Development of a Multiplex PCR Assay for Concurrent Detection of *Clavibacter michiganensis* ssp. *michiganensis* and *Xanthomonas axonopodis* pv. *vesicatoria*

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Abstract: In this study, concurrent amplification of *X. axonopodis* pv. *vesicatoria* and *C. michiganensis* ssp. *michiganensis* with primer pairs RST2-RST3 and CMM-5-CMM-6 was investigated by multiplex PCR. Reported CMM-5-CMM-6 and RST2-RST3 primer pairs were used to amplify template DNAs of *Clavibacter michiganensis* ssp. *michiganensis* and *Xanthomonas axonopodis* pv. *vesicatoria,* respectively. To optimize multiplex PCR reaction conditions; annealing temperature was determined by gradient PCR and primer concentrations for each primer pair were tested. Primer concentration ratios of 0.2 and 0.4 μM in final concentration for RST2-RST3 and CMM-5-CMM-6 primer pairs were found to be optimal for multiplex PCR reactions, respectively. Gradient PCR, in single and multiplex PCR reactions has shown that annealing temperatures 58.5-59.5°C were optimal for RST2-RST3 and CMM-5-CMM-6 primer pairs for multiplex PCR.

Key words: Bacterial canker, bacterial spot, tomato, multiplex PCR

INTRODUCTION

Clavibacter michiganensis ssp. *michiganensis* and *Xanthomonas axonopodis* pv. *vesicatoria* were important pathogens of tomato worldwide[1]. Seed borne nature of these pathogens necessitate the use of sensitive, reliable, fast and specific detection methods to prevent spread of diseases caused by these bacteria. DNA-based techniques such as Southern blot hybridization[2-4] and polymerase chain reaction (PCR)[2,5,6] were used for specific detection of *C. michiganensis* ssp. *michiganensis* and *Xanthomonas axonopodis* pv. *vesicatoria*. Dreier *et al.*[2] and Santos *et al.*[6] reported detection of *C. michiganensis* ssp. *michiganensis* from diseased tomato plants and seeds by PCR method. Primer pairs; CMM-5 and CMM-6, targeted to the plasmid-borne *pat-1* gene, produced a 614 bp amplification product[2]. Leite *et al.*[5] designed primers targeted to sequences of *hrp* genes of *Xanthomonas axonopodis* pv. *vesicatoria* that were able to distinguish non pathogenic xanthomonads from pathogenic ones by PCR.

PCR based methods are commonly used in detection and identification of pathogens for their reliable, fast and sensitive nature[7-12]. Multiplex PCR, a PCR technique, allows amplification of two or more target sequences simultaneously in a single reaction tube[13-15]. Multiplex PCR increases the efficiency of single PCR by saving time, labor and reducing reaction costs[13-18]. Four apple viruses were detected simultaneously by multiplex RT-PCR[18]. In olive, four olive viruses and *Pseudomonas savastanoi* pv. *savastanoi*, olive knot bacterial pathogen, were detected by multiplex RT-PCR[17]. Bacterial pathogens causing common and halo blights of bean were concurrently detected from bean seeds in a single PCR tube[16].

Despite the fact that multiplex PCR reduces time, labor and cost, annealing temperature differences of primer pairs, possible primer-primer dimers may reduce efficiency and reliability of PCR assays. Thus an optimization of multiplex PCR conditions is necessary before use in detection of more than one pathogen targets concurrently[13-15].

In this study, concurrent amplification of *X. axonopodis* pv. *vesicatoria* and *C. michiganensis* ssp. *michiganensis* with primer pairs RST2-RST3 and CMM-5-CMM-6 was investigated by multiplex PCR.

MATERIALS AND METHODS

Bacterial strains: *Clavibacter michiganensis* ssp. *michiganensis* ICMP (International Collection of Micro-organisms from Plants, Auckland, New Zealand) 2550 and *Xanthomonas axonopodis* pv. *vesicatoria* ICMP 9592 reference bacterial cultures were used in all studies. Bacterial cultures were grown in NBY (nutrient broth yeast extract)[19] for *C. michiganensis* ssp. *michiganensis* and YDC (yeast dextrose calcium carbonate)[20] for *X. axonopodis* pv. *vesicatoria*, at 24°C. Cultures were stored at -80°C in 30% glycerol for long term preservation. Cultures grown for 4-6 days were used in PCR assays.

PCR template preparation: DNA templates were prepared by boiling method. Three to four colonies were taken from 4-6 days old cultures and suspended in 200 μL of sterile distilled water and boiled for 15 min in Eppendorf thermomixer (Eppendorf AG, Hamburg, Germany). After boiling, templates were stored in refrigerator prior to PCR reaction preparations.

PCR primers: Primers RST2 (5AGGCCCTGGAA GGTGCCCTGGA3) and RST3 (5ATCGCACTGCG TACCGCGCGCG3) from *hrp* region of *X. axonopodis* pv. *axonopodis* producing a 840 bp[5] and CMM-5 (5GCGAATAAGCCCATATCAA3) and CMM-6 (5CGTCAGGAGGTCGCTAATA3) from plasmid-borne *pat-1* gene of *C. michiganensis* ssp. *michiganensis* producing a 614 bp amplification product[2] were used. Primers were synthesized by Genosys Co. (Sigma, St. Louis, MO, USA).

PCR reactions: PCR reactions were performed in Eppendorf thermocycler. Reactions contained 2.5 μL of 10X PCR buffer (100 mM Tris-HCl, pH 8.3, 500 mM KCl, Sigma), 200 μM dNTPs, 1.5 mM $MgCl_2$, 2 μL of template from boiled bacterial colonies and 0.05 μL of Taq polymerase (Fermentas, AB, Lithuania) in 25 μL volume. Sterile deionized water was used as negative control. Thermocycler conditions for primer concentration tests were 30 cycles of denaturing at 95°C for 30 sec, annealing at 59.4°C for 30 sec, extension at 72°C for 45 sec and final extension at 72°C for 5 min.

Amplification products were analyzed in 2% agarose gels run at 75 V for 40 min in 0.5 X TBE buffer and stained with ethidium bromide. Gels were photographed by Kodak EDAS 290 (Rochester, NY, USA) image system.

Gradient PCR: Annealing temperature gradients were determined to optimize annealing of primer pairs of RST2-RST3 and CMM-5-CMM-6 in Eppendorf gradient cycler. Temperatures from 57 to 61°C with 1°C increments; 57.4, 58.5, 59.5, 60.4 and 61.0°C, were tested for annealing temperature for multiplex PCR and single PCR conditions. This experiment repeated at least twice.

Primer concentrations: Primer concentrations in ratios of 0.1 (RST2-RST3):0.1 (CMM-5-CMM-6), 0.2:0.4 μM, 0.4:0.4 μM and 0.4:1.0 μM per final concentration were tested for RST2-RST3 and CMM-5-CMM-6 primer pairs, respectively. This experiment repeated twice.

RESULTS AND DISCUSSION

Annealing temperatures of primers of RST2-RST3 and CMM-5-CMM-6 for optimal amplification of *X. axonopodis* pv. *vesicatoria* and *C. michiganensis* ssp. *michiganensis* DNA templates for multiplex PCR were determined with single and multiplex gradient PCR under empirically chosen primer concentration of 0.4 μM (Fig. 1). Although annealing temperatures were given in studies of Dreier *et al.*[2] as 55°C for CMM-5 and CMM-6 and Leite *et al.*[5] as 62°C for RST2 and RST3 primers, single gradient PCR was used to determine the range of annealing temperatures for each pathogen that amplification occurs reliably. Thus, an annealing temperature for multiplex PCR can be determined according to both single and multiplex PCR reaction profiles. In single PCR, increasing annealing temperatures from 57.4 to 61°C produced a higher PCR band yield at 840 bp for *X. axonopodis* pv. *vesicatoria* template (Fig. 1, Lanes 1-5), on the contrary, at 614 bp, *C. michiganensis* ssp. *michiganensis* template amplification reduced with increasing temperatures (Fig. 1, Lanes 7-11). In multiplex PCR (Fig. 1, Lanes 13-17), at 57.4°C annealing temperature, both bands were detected at 840 and 614 bp. However, *X. axonopodis* pv. *vesicatoria* amplification was less efficient at this temperature. At 58.5°C, *C. michiganensis* ssp. *michiganensis* and *X. axonopodis* pv. *vesicatoria* were amplified efficiently.

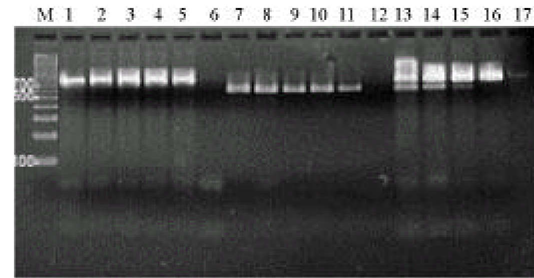

Fig. 1: Gradient PCR amplification of *X. axonopodis* pv. *vesicatoria* ICMP 9592 and *Clavibacter michiganensis* ssp. *michiganensis* ICMP 2550 in single and multiplex reactions. Lanes 1-6: Annealing temperatures tested for PCR amplification of *X. axonopodis* pv. *vesicatoria* with RST2-RST3 primers; 1: 57.4, 2: 58.5, 3: 59.5, 4: 60.4, 5: 61.0°C, 6: Water control. Lanes 7-12: Annealing temperatures tested for PCR amplification of *Clavibacter michiganensis* ssp. *michiganensis* with CMM-5-CMM-6 primers; 7: 57.4, 8: 58.5, 9: 59.5, 10: 60.4, 11: 61.0°C, 12: water control. Lanes 13-17: Annealing temperatures tested for multiplex PCR of *X. axonopodis* pv. *vesicatoria* and *Clavibacter michiganensis* ssp. *michiganensis* with primer pairs RST2-RST3 and CMM-5-CMM-6, respectively. 13: 57.4, 14: 58.5, 15: 59.5, 16: 60.4, 17: 61.0°C. M: 100 bp DNA ladder (Roche Diagnostics GmbH, Mannheim, Germany)

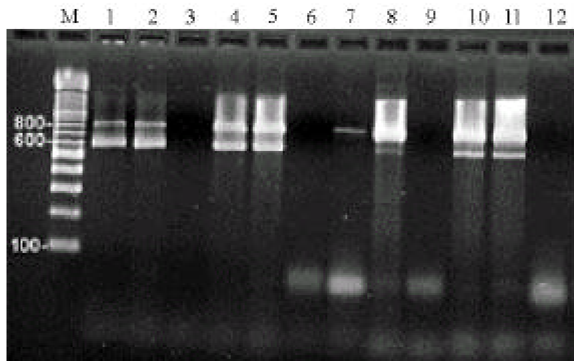

Fig. 2: Effects of primer concentration ratios of RST2-RST3 to CMM-5-CMM-6 primers in multiplex PCR amplification of *X. axonopodis* pv. *vesicatoria* and *C. michiganensis* ssp. *michiganensis*. Ratio of primer pairs of RST2-RST3 to CMM-5-CMM-6: Lanes 1 and 2: 0.1:0.4 μM, Lane 3: Water control (0.1:0.4 μM), Lanes 4-5: 0.2:0.4 μM, Lane 6: Water control (0.2:0.4 μM), Lane 7 and 8: 0.4:0.4 μM, Lane 9: Water control (0.4:0.4 μM), Lanes 10 and 11: 0.4:1.0 μM, Lane 12: Water control (0.4:1.0 μM). Two μL of DNA templates of *X. axonopodis* pv. *vesicatoria* strain ICMP 9592 and *C. michiganensis* ssp. *michiganensis* strain ICMP 2550 were used as templates prepared by boiling method. PCR reactions were performed in 25 μL volume. M: 100 bp DNA ladder (Roche), molecular sizes are given in base pairs.

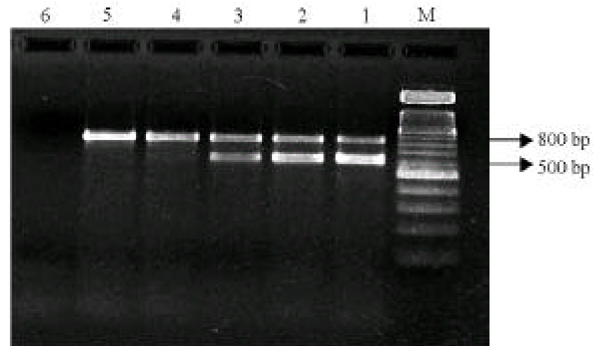

Fig. 3: Multiplex PCR amplification of *X. axonopodis* pv. *vesicatoria* ICMP 9592 (840 bp) and *C. michiganensis* ssp. *michiganensis* ICMP 2550 (614 bp) with RST2-RST3 and CMM-5-CMM-6 primers, respectively at annealing temperatures of 1: 57.4, 2:58.5, 3:59.5, 4:60.4, 5:61.0°C 6: Water control. M: 100 bp molecular marker (Roche). Primer concentrations were 0.2 μM for RST2-RST3 and 0.4 μM for CMM-5-CMM-6 primer pairs. Gel was run at 75 V for 40 min in 2% agarose (Sigma) in 0.5X TBE buffer

A weak band for *C. michiganensis* ssp. *michiganensis* was detected at 59.5°C and no PCR band was observed at 61°C. At 61°C, *X. axonopodis* pv. *vesicatoria* amplification showed a weak band though this result can be misinterpreted due to PCR tube was found to be not closed tightly after PCR. It was found that in duplicates of gradient PCR and in Fig. 3, an efficient amplification of *X. axonopodis* pv. *vesicatoria* was obtained at 61°C as in single gradient PCR (Fig. 1, Lane 5), but *C. michiganensis* ssp. *michiganensis* amplification was not reproducible at 60 and 61°C. Gradient PCR study showed that temperatures of 58.5-59.5°C were optimal for amplification of both primer pairs in multiplex PCR. It was found that *X. axonopodis* pv. *vesicatoria* template was more efficiently amplified with RST2-RST3 primers in comparison to *C. michiganensis* ssp. *michiganensis* amplification with CMM-5-CMM-6 primers.

After optimizing annealing temperature, in order to increase efficiency of *C. michiganensis* ssp. *michiganensis* amplifications, primer concentrations were tested in different ratios in multiplex PCR (Fig. 2). In tested ratios of RST2-RST3 to CMM-5-CMM-6 primers at 0.1: 0.4, 0.2:0.4, 0.4:0.4 and 0.4:1.0 μM concentrations have shown that 0.2:0.4 μM ratio (Fig. 2, Lanes 4-5) provided approximately equal amplification of both *C. michiganensis* ssp. *michiganensis* and *X. axonopodis* pv. *vesicatoria* even though the latter was slightly more efficiently amplified. At 0.1:0.4 μM primer concentrations, amplification of *C. michiganensis* ssp. *michiganensis* favored but *X. axonopodis* pv. *vesicatoria* amplification is significantly reduced (Fig. 2, Lanes 1-2). When the primer ratios were equal (0.4:0.4 μM) as was shown in Lane 8 and also this ratio was used in gradient PCR as mentioned above; *X. axonopodis* pv. *vesicatoria* amplification was significantly favored. In Lane 7, under same primer concentrations as in Lane 8, there was no amplification for *C. michiganensis* ssp. *michiganensis* and *X. axonopodis* pv. *vesicatoria* amplification was weak. This was probably due to PCR artifact that may be caused by not mixing DNA template well in PCR tube. At 0.4:1.0 μM primer concentration ratio, amplification of *X. axonopodis* pv. *vesicatoria* were more efficient than *C. michiganensis* ssp. *michiganensis* amplification even though CMM-5-CMM-6 primers were 2.5 times more concentrated than RST2-RST3 primers. This result indicates that after certain primer concentration level is reached for RST2-RST3 primers, increasing the primer concentrations of CMM-5-CMM-6 does not necessarily increase its amplification efficiency.

After determining optimal annealing temperature and primer concentration ratios, a multiplex gradient PCR was run at 0.2:0.4 μM (RST2-RST3 to CMM-5-CMM-6) primer concentrations (Fig. 3). Similar results were obtained as in

multiplex gradient PCR run under 0.4:0.4 μM primer concentrations (Fig. 1), however, at 0.2:0.4 μM primer concentration, PCR products at 840 and 614 bp were more clearly distinguishable for concurrent detection of *X. axonopodis* pv. *vesicatoria* and *C. michiganensis* ssp. *michiganensis* by multiplex PCR. In primer concentration assays, each primer concentration was run in duplicate in order to detect any false negatives resulting from crude DNA template preparation used in this study as boiling method. Except in Lane 7 (Fig. 2), PCR amplifications showed the same band pattern in duplicates. This probably can be caused by pipetting error. Two PCR reactions were performed to test possible binding of CMM-5 and CMM-6 primers with *X. axonopodis* pv. *vesicatoria* template or vice versa and no amplification was detected for *C. michiganensis* ssp. *michiganensis* template with RST2- RST3 primers and *X. axonopodis* pv. *vesicatoria* with CMM-5 and CMM-6 primers (data was not shown).

Results of this study show that coamplification of *X. axonopodis* pv. *vesicatoria* and *C. michiganensis* ssp. *michiganensis* templates in a single PCR tube can be performed using primer concentration ratios of 0.2:0.4 μM for RST2-RST3 and CMM-5- CMM-6 primers, respectively and annealing temperatures of 58.5 to 59.5°C. Detection of *X. axonopodis* pv. *vesicatoria* and *C. michiganensis* ssp. *michiganensis* from seeds by artificially inoculating tomato seeds and sensitivity of multiplex PCR can be further investigated.

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