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Early detection of *Clavibacter michiganensis* subsp. *Michiganensi* in tomato seedlings

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Abstract. The investigations of *Clavibacter michiganensis* subsp. *Michiganensi* in tomato seedlings were carried out using the BIO-PCR method. One to two (1–2) colonies were already detectable with species-specific primers CMM5 and CMM6 using *Clavibacter michiganensis* subsp. *michiganensis* bacteria in plant seedlings. The method allowed detection of bacteria at a distance of 8 cm farthest from the inoculation site in 95% of samples 3 days after treatment, at the earliest. Plant seedlings approximately 40 cm high were infected at full length after 9 days. Experimental results indicated the possibility of detecting the pathogen in very early stages of infection. Therefore, the BIO-PCR method was a highly specific, rapid and reliable detection technique, which might help to control the spread of the pathogen to healthy tomato plants.

Key words: bacterial canker, *Clavibacter michiganensis* subsp. *michiganensis*, tomato, BIO-PCR

INTRODUCTION

Clavibacter michiganensis subsp. *michiganensis* (Smith) Davis et al. (Cmm), the causal agent of bacterial canker of tomato (*Lycopersicon esculentum* Mill.), is an EPPO quarantine organism in Europe (EPPO/CABI, 1997). This disease spreads primarily among commercially grown tomatoes and causes major economic losses in Lithuania. It occurred in 80% of diseased tomato plants grown in some greenhouses last year (Burokienė, unpublished). Bacterial canker is one of the most difficult tomato diseases to control. The pathogen spreads through infected seeds and transplants. It is the way to spread Cmm bacteria to free areas (Chang et al., 1991; Gitaitis et al., 1991). Pathogen control is being carried out by using chemical (Dhanvantari, 1989; Florack et al., 1993; Werner et al., 2002) or physical treatment (Gleason et al., 1991) or biocontrol (Utkhede & Koch, 2004) on tomato seeds. Due to the economic importance of bacterial canker in tomato production, the application of new measures to control the agent of the disease was studied. In 2005, photosensitization was successfully applied as a novel biophotonic technique to direct inactivation of Cmm *in vitro*. This method is completely safe, reproducible, non-mutagenic, environmentally and human friendly and might be used as a new approach for pathogen control in seeds (Lukšienė et al., 2005). The other method involves detection and eradication of the pathogen in early stages of infection, because of the long period before the development of infection symptoms. The detection of the pathogen between infected or healthy seedlings at the time of transplanting is complicated, thus allowing undetected spread of Cmm. The control of bacteria spread in transplants usually is based on detection of

Cmm using sensitive serological (Franken et al., 1993) and DNA-based methods (Thompson et al., 1989; Ghedini & Fiore, 1995). The aim of the present investigation was to optimize the BIO-PCR method to detect Cmm bacteria in transplants during the early latent stage of infection.

MATERIALS AND METHODS

Bacterial strain. The reference strain of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm 8) obtained from Federal Centre for Breeding Research on Cultivated Plants (Institute of Resistance Research and Pathogen Diagnostics, Aschersleben, Germany) was used in these studies. Bacteria were grown on nutrient dextrose agar (NDA) and yeast glucose mineral agar (YGMA) medium at 25°C (Lelliott & Stead, 1987).

Inoculum preparation and inoculation. The inoculum of strain Cmm 8 was prepared from two-day-old bacterial culture on NDA slants. The bacteria were suspended in phosphate-buffered saline (PBS) (Na_2HPO_4 2.7 g, NaH_2PO_4 0.4 g, NaCl 8 g per 1 l H_2O_{dist}; pH 7.2). The inoculum concentration was adjusted to 10^8 cfu·ml^{-1} using spectrophotometer SEMCO S19E (EMCO, Poland) serial dilution plate method on NDA medium. The experiments were performed on five-week-old tomato seedlings cv. 'Monika'. The plants were inoculated with Cmm 8 strain by stabbing the stem above the first leaf with a needle previously dipped in the inoculum (Foster & Echandi, 1973). Inoculated plants were covered with polyethylene bags for 48 h and kept in a chamber at relative humidity of 85% within a 16/8h day/night photoperiod and temperature of 25/18°C.

DNA preparation for PCR. For DNA preparations, plants were cut into slices (2 cm) above the roots. The surfaces were disinfected by immersion in 70% ethyl alcohol, rinsed with sterile distilled water and air-dried. Every piece was crushed separately, added to 2 ml PBS and shaken 30 min at 25°C (rpm 100–150). The supernatant was transferred to the tube and centrifuged for 5–6 min at 8000 rpm. The pellet was diluted in 100 µl PBS and put on YGMA media at 25°C. After 2-3 days, bacteria were washed from the agar plate with 2–3 ml PBS and centrifuged for 10 min at 10,000 rpm. The pellet was diluted with 100–500 µl TE buffer and was kept 10 min in a water bath at 100°C and transferred to ice for 5 min. Suspension was diluted 50 times and 1 µl of DNA to PCR reaction was used. Tomato plants were tested after 1, 3, 6 and 9 days following inoculation. Five inoculated plants and two healthy plants as control were investigated in each case.

PCR procedures. PCR was carried out using primers CMM5 (5'-GCG AAT AAG CCC ATA TCA A-3') and CMM6 (5'-CGT CAG GAG GTC GCC TAA TA-3') specific for Cmm (Dreier et al., 1995). Amplification was performed in a total volume of 15 µl containing 1 µM of each primer DNA, 1 µl of a prepared DNA, 0.5 units of *Taq* DNA polymerase (Invitrogen, USA), 1.5 µM $MgCl_2$ and 0.2 mM of each dNTP, 10× PCR reaction buffer under the following reaction conditions: initial denaturation at 95°C for 3 min, 30 cycles of amplification – at 94°C for 30 s, at 55°C for 30 s and at 72°C for 1 min. The final elongation step was accomplished at 72°C for 5 min. Cmm amplifications were performed using T3000 Thermocycler (Biometra, Germany). Amplification products were analysed on 2% agarose gel stained in 0.5 µg·ml^{-1} ethidium bromide solution (Sambrook et al., 1989) using DNA size marker

GeneRuler 100 bp DNA Ladder Plus (Fermentas, Lithuania) and Transilluminator TI-1 (Biometra, Germany).

RESULTS AND DISCUSSION

The PCR assay was accomplished with the species-specific primers CMM5 and CMM6 (Dreier et al., 1995). The presence of bacteria was confirmed if amplification product (614 bp) was obtained in the samples of infected plant seedlings (Fig. 1). The amplification products were not obtained from DNA of bacteria of other subspecies or genera (data not shown).

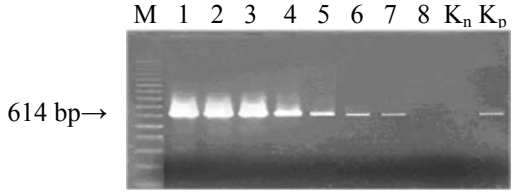

Fig. 1. Assessment of the PCR sensitivity threshold of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) in tomato seedlings. Lanes: M – DNA size marker; 1 – concentration of 4×10^8 cfu/ml; 2 – 4×10^7 cfu/ml; 3 – 4×10^6 cfu/ml; 4 – 4×10^5 cfu/ml; 5 – 4×10^4 cfu/ml; 6 – 4×10^3 cfu/ml; 7 – 4×10^2 cfu/ml; 8 – 4×10 cfu/ml; K_n – negative control; K_p – positive control.

The experiments were performed on five-week-old tomato seedlings cv. 'Monika' which average 40.7 cm in height. The plants were inoculated with Cmm 8 strain by stabbing the stem above the first leaf at the height of approximately 15 cm. One day later Cmm bacteria were detected in 100% of samples located only 2 cm from the inoculation site. Cmm bacteria were detected at a distance of 8 cm upwards and downwards from the inoculation site in 95% of samples 3 days after treatment, at the earliest. All samples of inoculated plants were infected at full length after 9 days. It was possible to detect Cmm 8 bacteria by BIO-PCR down to a concentration of 4×10^2 cfu·ml^{-1} (Fig. 1) in tomato seedlings. One to two(1–2) colonies of pathogen were already detectable in samples. Results showed that the BIO-PCR method was more sensitive and reliable for detecting Cmm as PCR experiments only (Ghedini & Fiore, 1995). Results indicated that it is possible to detect the spread of Cmm bacteria in seedlings at very early stages of infection. This method helps to restrict the spread of Cmm bacteria to healthy tomato plants. Furthermore, the method is very sensitive and reliable for controlling the agent of bacterial canker of tomato.

CONCLUSIONS

C. michiganensis subsp. *michiganensis* bacterium poses a direct threat to tomato production, which is one of the most important vegetables in Lithuania, economically. Control of the agent of bacterial canker ultimately depends on the accurate and sensitive detection of Cmm in infected tomato tissues in the early stages of infection. The BIO-PCR method using a primer set, CMM5 and CMM6, improved sensitivity of detection of 4×10^2 cfu·ml^{-1}. It was estimated that plants approximately 40 cm high were already systemically infected after 9 days. The presented data enables the detection of 1-2 colonies of Cmm by a method which is completely safe to the

environment and friendly to humans,and can help to reduce the spread of the disease agent to healthy plants or to areas free from pathogen.

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