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Clavibacter michiganensis subsp. *michiganensis*, Bacterial Canker of Tomato: 2. Comparison of the Effectiveness of Extraction Procedures and Sensitivity of Methods for Detection in Tomato Seeds

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SUMMARY

Two seed extraction procedures, used for detection of ***Clavibacter michiganensis*** subsp. *michiganensis* (*Cmm*) in artifficially infested tomato seed lots, were evaluated. A comparison of the efficiency of pathogen detection by using different extraction methods showed that a grinding procedure was more effective than soaking seed samples. The extraction by grinding resulted in a higher number of samples with *Cmm* colonies than did the method that included soaking. The detection threshold of *Cmm* in relation to seed sample size was evaluated by adding different numbers of artificially infested seeds to uninfected samples of 2000 or 5000 seeds. Four detection methods were simultaneously compared for their sensitivity in *Cmm* detection in seeds: isolation on semiselective media (mSCM, D_2ANX, mCNS), direct PCR from seed material, Bio-PCR with initial culturing of bacteria on NBY agar prior to PCR, and Enrichment PCR. The pathogen was detected in samples of 2000 seeds containing one, five and ten infested seeds, in at least two out of three replicates by three detection methods (selective plating, direct PCR and Bio-PCR), using the grinding extraction method with an addition of centrifugation step. In samples of 5000 seeds, five infested seeds were detected in all replicates by the same detection methods. Similar results were obtained by the soaking extraction method. In Enrichment PCR, positive results were obtained only in samples of 2000 seeds containing five and ten infested seeds regardless of the extraction method.

Keywords: Bacterial canker; Bacterial wilt; Tomato seeds; Extraction procedure; Detection threshold; Selective media; PCR

INTRODUCTION

Bacterial canker is one of the most serious tomato diseases, causing severe losses both in greenhouse and open-field tomato production. It is caused by the bacterium *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), which is widespread in main tomato growing areas in the world (Chang et al., 1992; EPPO/CABI, 2005). The pathogen is seed-borne and persists in plant debris in soil and on contaminated greenhouse structures. Its long distance dissemination is feasible by contaminated seed and infested soil. Secondary spread is accomplished by means of splashing water, contaminated equipment and cultural practices. Infected seed is often considered to be the primary source of inoculum, as well as a major source of *Cmm* infection outbreaks (Tsiantos, 1987). Thus, a few infected seedlings in a seed bed can initiate severe infection in the field or the greenhouse. Considering the seed-borne nature of *Cmm*, the bacterium usually enters a production area mainly through infested seeds (Strider, 1969) or latently infected tomato transplants (Gittaitis et al., 1991). Therefore, indexing of tomato seeds for the canker pathogen is an important step in disease control and prevention of outbreaks. However, there are several problems in seed screening for the canker pathogen. In seed health testing, identification of the pathogen is often complicated by the presence of fast-growing saprophytes that resemble *Cmm* in colony morphology. Consequently, numerous selective media for *Cmm* isolation have been developed (Gross and Vidaver, 1979; Chun, 1982; Fatmi and Schaad, 1989; Bolkan et al., 1996). Low population levels of the bacterium in/on seed also make its detection by conventional methods very difficult. PCR-based procedures have therefore been developed (Dreier et al., 1995; Santos et al., 1997; Pastrik and Rainey, 1999). Although considerable efforts have been made to improve the efficiency and sensitivity of assays for *Cmm* detection in commercial tomato seeds and seedlings (Fatmi and Schaad, 1988; Gittaitis et al., 1991), fast and accurate methods are continually sought to simultaneously identify and differentiate the pathogen. However, the variable levels of seed infection and broad morphological variation among *Cmm* strains increase the difficulty in standardizing an appropriate detection method with uniform sensitivity and reproducibility (Hadas et al., 2005).

Therefore, the objectives of this study were to evaluate several seed extraction procedures used for the detection of *Cmm* in seed lots and to determine the detection threshold of *Cmm* in relation to seed sample size using different methods (isolation on several selective media, direct PCR, Bio-PCR and Enrichment PCR).

MATERIAL AND METHODS

Bacterial strains and different selective media

Bacterial strains used in this study are listed in Table 1. Reference strain of *C. michiganensis* ssp. *michiganensis* CFBP 4999 (equivalent strain designation = NCPPB 2979) obtained from Collection Française des Bactéries Phytopathogènes (CFBP) was also included in the experiments. Bacteria were maintained on plates containing nutrient agar (NA) and incubated at 27°C for 48 h. Short period storage was carried out at 4°C on King's B medium (KBM) slants and for long periods in sterile destilled water in Eppendorf tubes at room temperature.

The following media were used:

- mSCM (Bolkan et al., 1996) containing (per liter): 0.1 g yeast extract, 10 g mannose, 1.5 g H_3BO_3 (boric acid), 0.25 g $MgSO_4 x 7H_2O$, 2 g K_2HPO_4, 1.5 g KH_2PO_4, 15 g agar; after autoclaving add: 30 mg nalidixic acid (10 mg/ml in 0.1M NaOH), 100 mg nicotinic acid (20 mg/ml in distiled H_2O) and 200 mg cycloheximide (200 mg/ml in absolute methanol);
- D_2ANX (Chun, 1982) containing (per liter): 10 g glucose (dextrose), 4 g casein acid hydrolisate, 2 g yeast extract, 1 g NH_4Cl, 0.3 g $MgSO_4 x 7H_2O$, 1.2 g trizma base, 15 g agar; after autoclaving add: 30 mg nalidixic acid (10 mg/ml in 0.1M NaOH), 100 mg cycloheximide (200 mg/ml in absolute methanol) and 10 mg polymixin B sulfate (10 mg/ml in distilled H_2O);
- mCNS (Gross and Vidaver, 1979) containing (per liter): 5 g peptone, 3 g yeast extract, 0.5 g KH_2PO_4, 2 g K_2HPO_4, 0.25 g $MgSO_4 x 7H_2O$, 5 g LiCl, 15 g agar; after autoclaving add: 30 mg nalidixic acid (10 mg/ml in 0.1M NaOH), 50 mg cycloheximide (200 mg/ml in absolute methanol) and 38 mg polymixin B sulfate (10 mg/ml in distilled H_2O);
- NBY (Vidaver, 1967) containing (per liter): 16 g nutrient broth, 4 g yeast extract, 4 g KH_2PO_4, 4 g K_2HPO_4, 0.246 g $MgSO_4 x 7H_2O$, 5 g glucose (dextrose), 15 g agar;

- CBM (Hadas et al., 2005) is a modification of NBY containing (per liter): 16 g nutrient broth, 4 g yeast extract, 4 g KH_2PO_4, 4 g K_2HPO_4, 0.246 g $MgSO_4$ x $7H_2O$, 5 g glucose (dextrose), 15 g agar; after autoclaving add: 25 mg nalidixic acid (10 mg/ml in 0.1M NaOH), 200 mg cycloheximide (200 mg/ml in absolute methanol), 32 mg polymixin B sulfate (10 mg/ml in distilled H_2O).

All medium heat-stabile components were dissolved in distilled water by steaming and then autoclaved for 15 minutes at 121°C during 20 minutes. Heat-labile components (antibiotics) were filter-sterilized and added to molten autoclaved medium previously cooled to 55°C and then poured into 9 cm Petri dishes.

In order to select the most suitable media for isolation of *Cmm* from seeds, four strains obtained from the samples collected in different regions in Serbia (P-2, P-4, P-69, P-71) and the reference strain CFBP 4999 were inoculated on four selective media (mSCM, D_2ANX, mCNS, and CBM) and their growth was compared with that on the nonselective NBY agar. The plates were inoculated by spreading 100 µl of bacterial suspension prepared from tested strains (concentrations adjusted to approximately 10^2 and 10^3 cfu/ml) and incubated at 27°C for 4-11 days. For each media and both dilutions, three replicates were used. The media which supported the growth of chosen strains and easy characterization of *Cmm* strains were selected for further testing of additional fifteen strains (Table 1). Colonies that exhibited colony morphology typical for *Cmm* on different selective media, compared to the reference strain CFBP 4999, were subsequently purified and identified based on Gram reaction and polymerase chain reaction (PCR) as described below.

Seed inoculation

Infested seeds were prepared according to a method given by Hadas et al. (2005) as follows: a culture of *Cmm* strain P-8 grown at 27°C on NBY for 48 h was suspended in sterile distilled water and concentration of bacterial suspension adjusted to 10^8 cfu/ml using Mc-Farland scale and confirmed by dilution plating (Klement et al., 1990). A seed lot (1000 seeds) was then infiltrated under pressure of 100 kPa in 10 ml of bacterial suspension for 5 minutes. The infiltrated seeds were dried on filter paper in laminar-flow chamber for 24 h, and then placed into a dessicator and kept for three weeks at room temperature. To determine the amount of *Cmm* in an individual infested seed, each of the 10 artificially inoculated seeds were placed in pestile and ground with mortar in 0.6 ml 0.1M PBS (phosphate buffer and saline, containing per liter: 7.75 g Na_2HPO_4, 1.65 g KH_2PO_4, 0.5 g ascorbic acid, 8 g NaCl, pH was adjusted to 7.4 after autoclaving), and 100 µl of the extract was plated on each of the three NA (nutrient agar) plates. The infested seeds were used in experiments to determine the most appropriate seed extraction procedure and detection threshold of *Cmm* in relation to seed sample size.

Extraction methods

Two extraction procedures were evaluated with samples of 2000 (≈ 5 g) or 5000 seeds (≈ 12.5 g), cv. Saint Pierre. The procedures included: (1) grinding with a mill and (2) soaking in PBS buffer. In procedure 1) dry seeds were ground with an electric mill and then suspended in 35 or 70 ml, respectively, of sterile PBS supplemented with 0.1% agar (w/v). The suspension was shaken for an hour and then kept at 4°C for at least 16 hours. Additionally, ground samples were centrifuged at 6000 g for 10 minutes and the pellets were resuspended in 1 ml of sterile PBS. To avoid cross-contamination between samples, the grinding mill was disinfected by spraying with 96% ethanol and drying. In procedure (2) the seeds were suspended in 28 or 70 ml, respectively, of sterile PBS buffer supplemented with 0.1% agar, shaken for an hour and kept at 4°C for at least 16 hours. Serial 10-fold dilutions in PBS were prepared and 100 µl of each dilution plated on mSCM, D_2ANX, mCNS and NBY media in Petri dishes. After incubation at 27°C for 4, 5, 6 and 11 days on NBY, D_2ANX, mCNS and mSCM, respectively, suspected *Cmm* colonies were recorded, purified and subjected to Gram staining and PCR as described below.

To compare the effectiveness of the two described extraction procedures, seed lots with different infestation levels were used. Lots 1-4 had sample size of 2000 seeds. Infested seeds were not added to lot 1. Lots 2, 3 and 4 contained one, five and ten artificially infested seeds, respectively. Lots 5 and 6 that had sample size of 5000 seeds, contained one and five infested seeds, respectively. Seed samples were then subjected to both extraction methods and serial dilutions were plated on mSCM, D_2ANX, mCNS and NBY media. Colonies that developed were confirmed by Gram staining and PCR. The experiment was repeated twice with three replicates on

three different media for each infestation level (total of nine replicates). The ratio between the number of replicate agar plates with and without *Cmm* colonies from a total of nine replicates was calculated for each extraction method (Hadas et al., 2005). The results of each experiment were considered separately. The data were subjected to t-test at $P < 0.05$ to determine statistical significance between mean values of the parametars measured.

Determination of detection threshold of *Cmm* in relation to seed sample size

The detection threshold, evaluated by adding different numbers of artificially infested seeds to uninfected samples of 2000 and 5000 seeds, and extraction were done using both procedures described above.

Four detection methods were simultaneously compared for their sensitivity in *Cmm* detection in seeds: isolation on selective media (mSCM, D_2ANX, mCNS and NBY as described above), direct PCR from seed material, Bio-PCR with initial culturing of bacteria on NBY agar prior to PCR, and Enrichment PCR according to EPPO PM 7/42 (1) procedure.

Pathogenicity tests

Pathogenicity tests on tomato seedlings were performed as described in details (Milijašević et al., 2006). Seedlings were inoculated at the second true leaf stage for each strain, including the reference one, by injection into the stem at the cotyledons, and kept under plastic bags for 48 hours. The plants injected with water served as negative control. The plants were maintained at 26°C and observed for wilting and stem canker for another twenty days.

Polymerase chain reaction tests – direct PCR, Bio-PCR and Enrichment-PCR

All PCR reactions were performed in Eppendorf thermocycler. Primers were synthesized by Fermentas (Lithuania). PCR amplifications were carried out with primers CMM-5, CMM-6 using the PCR protocol of Drier et al. (1995) and primers PSA-4, PSA-R according to the PCR protocol of Pastrik and Rainey (1999), as previously described in details (Milijašević et al., 2006).

The DNA amplifications were performed in a total volume of 25 μl. The reactions contained: 1 X PCR Master mix (Fermentas, Lithuania) (0.625 U Taq polymerase, 2 mM $MgCl_2$, 0.2 mM each dNTPs), 1 μl of each primer (20 μM) and 2.5-10 μl of template DNA. The reference strain of *C. michiganensis* subsp. *michiganensis* (CFBP 4999) was used as positive control for all PCR amplifications and sterile deionized water was used as negative control. A second negative control, consisting of samples without infested seeds, was included in the direct and Enrichment PCR. To test the inhibition effect of seed material, a second positive control containing both seeds and CFBP 4999 was also considered.

For direct PCR amplification, DNA extraction from seed material based on a protocol described by Edwards et al. (1991) was performed. Aliquots of 250 μl of seed extract were placed in 750 μl of extraction buffer (200 mmol/L Tris HCl, pH 7.5; 250 mmol/L NaCl; 25 mmol/L EDTA; 0.5% SDS; 2% polyvinyl pyrrolidone (PVP)) and shaken for 30 min at room temperature. Then they were centrifuged at 1850 g for 2 min and 300 μl of the supernatant fluids were taken and heated for 5 min at 93°C. The samples were then centrifuged at 12000 g; 200 μl of each supernatant fluid were collected and 200 μl isopropanol were added, mixed gently, and left for an hour at room temperature. The mixture was centrifuged at 12000 g for 15 min, the supernatant fluid was removed, the precipitate was dried and resuspended in 100 μl sterile ultrapure water. For direct PCR 10 μl were used as template DNA. For Bio-PCR, 100 μl of the seed extract were spread onto NBY medium and incubated at 27°C for 48 h. To prepare a template DNA a single colony was suspended in 100 μl of sterile distilled water in a microvial. Closed vials were heated at 95°C for 15 minutes, cooled on ice and pulse centrifuged. A 2.5 μl aliquot of the bacterial suspension was added to PCR reaction as a template DNA. For Enrichment-PCR, 500 μl of the seed extract was transferred into a test tube filled with 5 ml of sterile liquid semi-selective medium for isolation of *Cmm* from tomato seeds (Dhanvantari, 1988) containing (per liter): 5 g proteose peptone No 3, 5 g bacto trypton, 10 g mannose, and 1.5 g boric acid. No antibiotics were added to the liquid medium. Test tube inoculated with cell suspension of the reference *Cmm* strain, at contamination level 10^3 cfu/ml, was used as positive control. The enrichment tubes were incubated at 27 °C for 72 h. Aliquots of 100 μl from each enrichment tube were transferred into a sterile microvial and heated at

95°C for 15 minutes, cooled on ice and pulse centrifuged. A 10 μl aliquot was added to PCR reaction as a template DNA. For Enrichment PCR, only the protocol by Pastrik and Rainey (1999) was used, being the only validated protocol for the seed test. The amplified products were subjected to electrophoresis in 2% agarose gel run at 100 V for 30 minutes in TBE buffer and stained with ethidium bromide.

RESULTS

Characterization and recovery of *Cmm* strains on different media

All strains listed in Table 1 were identified as *Cmm* according to their morphology on selective media, pathogenicity tests, Gram reaction (+) and PCR tests using both primer sets (CMM-5 + CMM-6 and PSA-4 + PSA-R). The bacterial colonies on the NBY were smooth, yellow, mucoid, circular, convex with entire margins, 2-3 mm in diameter after 3 days. On D_2ANX colonies were light yellow, mucoid, circular, convex, and smaller in diameter than colonies on NBY (2-3 mm after 6 days). The colonies on NBY and D_2ANX after 11 days were shown on Figure 1 and Figure 2, respectively. All strains were circular, convex, gray in colour and very small in diameter (1-2 mm after 11 days) on mSCM medium (Figure 3). On mCNS and CBM media, strains were also circular, deep yellow and very small (2-3 mm after 11 days) (Figures 4 and 5).

CBM poorly supported the growth of most strains and therefore it was discarded from further testing. The recovery of *Cmm* strains on the three chosen selective

Figure 1. *Cmm* colonies on NBY media after 11 days
Slika 1. Kolonije *Cmm* na NBY podlozi nakon 11 dana

Table 1. Percentage recovery of *Cmm* strains on different selective media in comparison to NBY media
Tabela 1. Odnos (%) broja kolonija *Cmm* na različitim selektivnim podlogama u poređenju sa brojem na NBY podlozi

Strain Soj	Media – Podloge			PCR primer sets – Parovi prajmera	
	mSCM	D_2ANX	mCNS	CMM-5+CMM-6	PSA-4+PSA-R
P-2	0	1.4	59.9	+	+
P-4	63.2	9.9	190.9	+	+
P-5	20.0	166.0	109.4	+	+
P-7	88.9	204.0	75.1	+	+
P-8	30.1	83.0	34.5	+	+
P-10	37.5	82.2	55.8	+	+
P-12	155.1	176.7	88.2	+	+
P-13	18.8	35.6	75.3	+	+
P-16	40.0	80.0	42.4	+	+
P-17	42.8	77.5	80.8	+	+
P-18	119.8	106.3	108.5	+	+
P-67	0.75	84.6	59.2	+	+
P-68	48.1	104.3	98.8	+	+
P-69	125.7	128.6	105.1	+	+
P-70	88.6	54.6	19.6	+	+
P-71	134.8	106.1	109.6	+	+
P-72	0	76.4	54.6	+	+
P-73	17.5	47.6	64.4	+	+
P-74	43.6	61.1	45.7	+	+
CFBP 4999	124.7	131.8	140.2	+	+

Figure 2. *Cmm* colonies on D_2ANX media after 11 days
Slika 2. Kolonije *Cmm* na D_2ANX podlozi nakon 11 dana

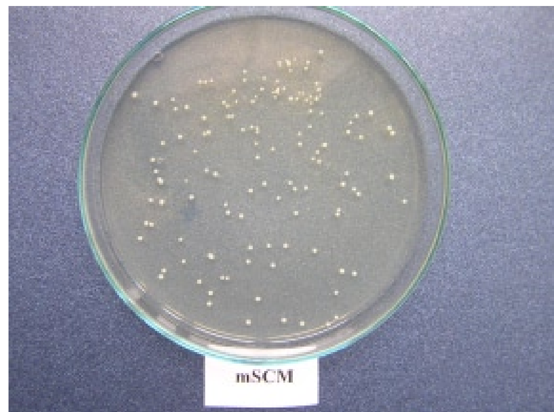

Figure 4. *Cmm* colonies on mSCM media after 11 days
Slika 4. Kolonije *Cmm* na mSCM podlozi nakon 11 dana

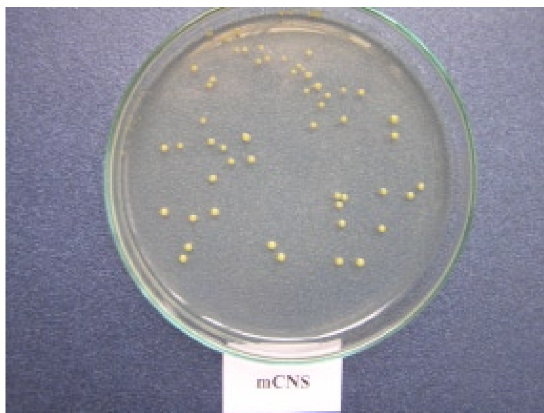

Figure 3. *Cmm* colonies on mCNS media after 11 days
Slika 3. Kolonije *Cmm* na mCNS podlozi nakon 11 dana

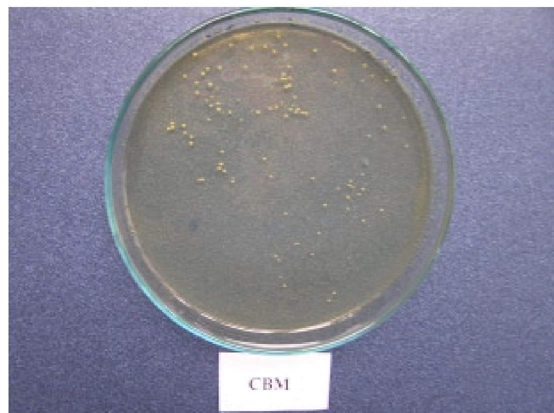

Figure 5. *Cmm* colonies on CBM media after 11 days
Slika 5. Kolonije *Cmm* na CBM podlozi nakon 11 dana

media (mSCM, D_2ANX, mCNS) appeared to be highly variable (Table 1). For example, strain P-2 grew very well on mCNS but did not grow on mSCM and grew poorly on D_2ANX. Strain P-72 did not grow at all on mSCM but grew on D_2ANX and mCNS. However, each of the tested strains grew on at least two of the selective media. D_2ANX and mCNS enabled growth of all tested strains, although the recovery was higher on D_2ANX. Although two strains failed to grow on mSCM, the total recovery of *Cmm* strains on that medium was abundant. These three media were subsequently used for comparisons of different extraction procedures and determination of detection threshold along with PCR based methods.

Infested seeds

The amount of *Cmm* on individual artificially infested seed was determined with 10 seeds for the inoculum level of 10^8 cfu/ml. It ranged between 5 and 6 x 10^8 cfu/ml per seed.

Comparison of the effectivness of extraction procedures for detection of *Cmm* in tomato seeds

Lot 1, which was used as control and was considered to be pathogen-free, did not contain *Cmm*. A comparison of the pathogen detection efficiency by using two extraction procedures showed that grinding was more efficient than soaking. Extraction by grinding resulted in a slightly higher number of samples with *Cmm* colonies in the lots containing five infested seeds in the sample of 5000 seeds in the first experiment (Figure 6), and one and five infested seeds in the samples of 2000 seeds in the second experiment (Figure 7).

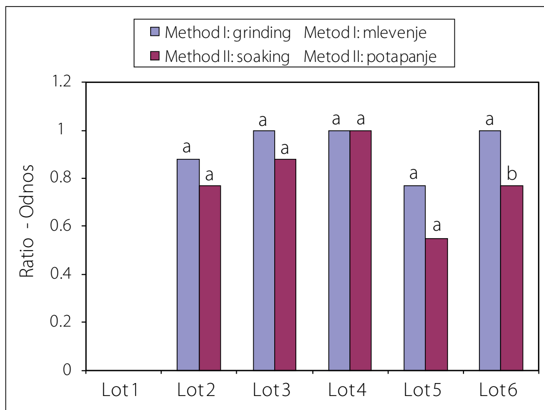

Figure 6. Ratio of replicate agar plates with positive colonies of *Cmm* from a total of nine inoculated replicate plates for both extraction procedures (experiment 1)
Lot 1 = control (2000 seeds) without infested seeds added
Lot 2 = one infested seed added per 2000 seeds
Lot 3 = five infested seeds added per 2000 seeds
Lot 4 = ten infested seeds added per 2000 seeds
Lot 5 = one infested seed added per 5000 seeds
Lot 6 = five infested seeds added per 5000 seeds
Slika 6. Odnos broja petri-kutija sa podlogama na kojima su registrovane kolonije *Cmm* od ukupno devet ponavljanja za obe procedure ektrakcije (ogled 1)
Uzorak 1 = kontrola (2000 semena) bez dodatka zaraženih semena
Uzorak 2 = dodato jedno zaraženo seme u 2000 semena
Uzorak 3 = dodato pet zaraženih semena u 2000 semena
Uzorak 4 = dodato deset zaraženih semena u 2000 semena
Uzorak 5 = dodato jedno zaraženo seme u 5000 semena
Uzorak 6 = dodato pet zaraženih semena u 5000 semena

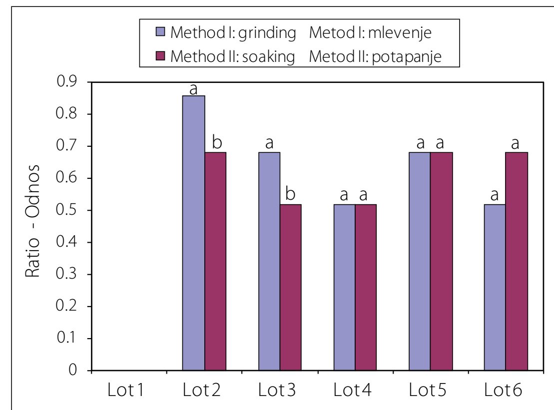

Figure 7. Ratio of replicate agar plates with positive colonies of *Cmm* from a total of nine inoculated replicate plates for both extraction methods (experiment 2)
Lot 1 = control (2000 seeds) without infested seeds added
Lot 2 = one infested seed added per 2000 seeds
Lot 3 = five infested seeds added per 2000 seeds
Lot 4 = ten infested seeds added per 2000 seeds
Lot 5 = one infested seed added per 5000 seeds
Lot 6 = five infested seeds added per 5000 seeds
Slika 7. Odnos broja petri-kutija sa podlogama na kojima su registrovane kolonije *Cmm* od ukupno devet ponavljanja za obe procedure ektrakcije (ogled 2)
Uzorak 1 = kontrola (2000 semena) bez dodatka zaraženih semena
Uzorak 2 = dodato jedno zaraženo seme u 2000 semena
Uzorak 3 = dodato pet zaraženih semena u 2000 semena
Uzorak 4 = dodato deset zaraženih semena u 2000 semena
Uzorak 5 = dodato jedno zaraženo seme u 5000 semena
Uzorak 6 = dodato pet zaraženih semena u 5000 semena

Determination of detection threshold of *Cmm* using different methods in relation to seed sample size

In samples of 2000 seeds containing one, five and ten infested seeds, the pathogen was detected in at least two out of three replicates by three detection methods (selective plating, direct PCR and Bio-PCR), using grinding extraction method with an addition of centrifugation step. In samples of 5000 seeds, five infested seeds were detected in all replicates by the same detection methods (Table 2). Similar results were obtained by the soaking extraction method (Table 3). In Enrichment PCR, positive results were obtained only in samples of 2000 seeds containing five and ten infested seeds, regardless of the extraction method.

DISCUSSION

Infected tomato seeds are often considered to be the primary source of inoculum for bacterial canker infections, pathogen introduction and outbreaks of the disease in new regions. Moreover, field studies in the United States have shown that a single infected seed in 10000 can initiate an epidemic under favourable conditions (Gitaitis et al., 1991). As a result, numerous attempts have been made to develop and standardize protocols for detection of the pathogen in seeds (Fatmi and Schaad, 1988; Biggerstaff et al., 2000; Hadas et al., 2005).

Several semiselective media for isolating *Cmm* have been developed (Schaad et al., 2001). It is generally known that *Cmm* strains exhibit broad variation in colony morphology, even on the same medium. Therefore, it is advisable to use several different media simultaneously. In the present study, four dif-

Table 2. The detection threshold of *Clavibacter michiganensis* subsp. *michiganensis* (seed extraction method with 2000 and 5000 seeds: grinding)
Tabela 2. Prag detekcije *Clavibacter michiganensis* subsp. *michiganensis* (metod ekstrakcije mlevenjem u uzorcima veličine 2000 i 5000 semena)

Infested seed/sample Zaražena semena/uzorak	Detection method – Metod detekcije			
	Plates (mSCM, D_2ANX, mCNS) Podloge (mSCM, D_2ANX, mCNS)	Direct PCR Direktni PCR	Bio-PCR	Enrichment PCR
0/2000	0/3	0/3	0/3	0/3
1/2000	2/3	2/3	3/3	0/3
5/2000	3/3	3/3	3/3	3/3
10/2000	3/3	3/3	3/3	3/3
1/5000	2/3	0/3	3/3	0/3
5/5000	3/3	3/3	3/3	0/3

Table 3. The detection threshold of *Clavibacter michiganensis* subsp. *michiganensis* (seed extraction method with 2000 and 5000 seeds: soaking)
Tabela 3. Prag detekcije *Clavibacter michiganensis* subsp. *michiganensis* (metod ekstrakcije potapanjem u uzorcima veličine 2000 i 5000 semena)

Infested seed/sample Zaražena semena/uzorak	Detection method – Metod detekcije			
	Plates (mSCM, D_2ANX, mCNS) Podloge (mSCM, D_2ANX, mCNS)	Direct PCR Direktni PCR	Bio-PCR	Enrichment PCR
0/2000	0/3	0/3	0/3	0/3
1/2000	2/3	2/3	3/3	0/3
5/2000	3/3	3/3	3/3	3/3
10/2000	3/3	3/3	3/3	3/3
1/5000	1/3	0/3	3/3	0/3
5/5000	3/3	3/3	3/3	0/3

ferent semiselective media were examined with nineteen *Cmm* strains originating from different regions in Serbia. Three media (mSCM, D_2ANX and mCNS) were selected for further studies since they provided the best growth of most strains tested.

The sensitivity of assays for bacteria detection in seeds depends on the extraction method (Roth, 1989). The results of our study demonstrated that the extraction of *Cmm* in seeds by grinding was slightly more effective than by soaking. However, from a practical point of view, the grinding method is limited in sample size and makes the tested seeds useless for further purposes. Therefore the development of nondestructive seed assays for *Cmm* detection should be considered.

The sensitivity of different detection methods depends on several other factors: variation in the levels of seed contamination, procedures used to extract bacteria from the seeds and infection rates of seeds (Hadas et al., 2005). For that reason, different results have been obtained concerning *Cmm* detection threshold in tomato seeds. For example, the SCM medium developed by Fatmi and Schaad (1988) is claimed to detect a single infected seed in samples of 10000 seeds. On the other hand, in the study conducted by Hadas et al. (2005), it was shown that one infected seed in a sample of 10000 could be detected only by Bio-PCR, and in only one out of five replicates. The authors were also able to detect one infected seed among 5000 seeds in all subsamples by agar plating after adding a concentration step, and both by direct and by Bio-PCR. However, in the present study, it was shown that one infected seed in samples containing 5000 seeds could only be detected by Bio-PCR and agar plating, while negative results were obtained for direct and Enrichment PCR in spite of the fact that DNA extraction was done from the samples before conducting PCR. Generally, the pathogen was detected by direct PCR in samples of 2000 seeds containing one, five and ten infested seeds, and in samples of 5000 seeds containing five infested seeds in at least two out of three replicates, but it should also be noted that in our pre-

vious experiments (data not shown) negative results were obtained for direct PCR when DNA extraction was not performed. Although PCR-based methods are usually considered more sensitive than other detection methods (Louws et al., 1999), the results of our study, similar to those obtained by Hadas et al. (2005), showed that only Bio-PCR was more sensitive than dilution plating on semiselective media. This is probably due to the low levels of contamination by saprophytic bacteria in the seed lots used in this study. Under different circumstances in highly contaminated seed lots, direct PCR method would probably have an advantage over the agar plating assays.

It should also be emphasized that the general recommendation by ISTA for standard sample size is 10000 seeds in order to detect 0.03% contamination level in a seed lot (OEPP/ EPPO, 2005). The sample is therefore usually devided into subsamples, each containing not more then 2000 seeds. Hadas et al. (2005) concluded that the detection of one infected seed required a test sample not larger than 5000 seeds. However, the results of our study suggested that smaller samples (1000 or 2000 seeds) would be appropriate considering the available extraction procedures and detection methods. In the present study, the best results were obtained with selective plating and a combination of sample pre-incubation on selective or common media and PCR, i.e. Bio-PCR. Direct PCR proved to be a sensitive method but less efficient, and it depended on DNA extraction from the seed material, probably due to a presence of substances that inhibited Taq polymerase in the plant material. Therefore, looking from the aspect of the available detection methods of *Cmm* in seeds, we suggest the use of samples not larger than 2000 seeds and a combination of two different detection methods: simultaneous plating on at least two semiselective media and Bio-PCR or direct PCR, for testing of tomato seeds for the canker pathogen. However, these results should be confirmed by testing several naturally infected seed lots from various sources.

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Clavibacter michiganensis subsp. *michiganensis,* bakteriozni rak paradajza: 2. Poređenje efikasnosti procedura za ekstrakciju i osetljivosti metoda za detekciju na semenu paradajza

REZIME

Ocenjivane su dve procedure ekstrakcije koje se primenjuju u detekciji *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) u uslovima veštačke inokulacije uzoraka semena paradajza. Poređenje efikasnosti detekcije patogena korišćenjem metoda mlevenja i potapanja semena pokazalo je da je metod mlevenja bio efikasniji. Ekstrakcija mlevenjem semena rezultirala je neznatno većim brojem uzoraka sa kolonijama *Cmm* nego procedura potapanja semena. Prag detekcije *Cmm* u zavisnosti od veličine uzorka ocenjivan je dodavanjem različitog broja veštački inokulisanih semena u nezaražene uzorke semena veličine 2000 i 5000 semena. Istovremeno je poređena osetljivost četiri metode za detekciju *Cmm* na semenu: izolacija na poluselektivne podloge (mSCM, D_2ANX, mCNS), direktni PCR iz uzoraka semena, Bio-PCR koji uključuje gajenje bakterija na NBY podlozi pre PCR reakcije i Enrichment PCR. Patogen je detektovan u uzorcima veličine 2000 semena u koje su dodati jedno, pet i deset veštački zaraženih semena, u najmanje dva od tri ponavljanja korišćenjem tri metode za detekciju (izolacija na poluselektivne podloge, direktni PCR i Bio-PCR), nakon ekstrakcije mlevenjem uzoraka. Istim metodama, u uzorcima veličine 5000 semena, pet zaraženih semena je detektovano u svim ponavljanjima. Slični rezultati su dobijeni i nakon ekstrakcije potapanjem semena. U Enrichment PCR reakciji, pozitivni rezultati su dobijeni samo u uzorcima veličine 2000 semena u koje je dodato pet i deset zaraženih semena nezavisno od toga koja je procedura ekstrakcije korišćena.

Ključne reči: Bakteriozni rak; bakteriozno uvenuće; seme paradajza; procedure ekstrakcije; prag detekcije; selektivne podloge; PCR