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Comparison of extraction procedures and determination of the detection threshold for *Clavibacter michiganensis* ssp. *michiganensis* in tomato seeds

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Several seed extraction procedures, used for detection of *Clavibacter michiganensis* ssp. *michiganensis* (*Cmm*) in naturally infected and artificially infested tomato seed lots were evaluated. Extraction methods that included grinding the seeds were significantly better at detecting the pathogen in three different seed lots than methods that used only soaking. The detection threshold of *Cmm* in relation to seed sample size was determined by adding naturally infected seeds into samples of three different sizes. *Cmm* was detected by agar plating assay, on three media (CNS, mSCM, D_2ANX), and by direct PCR from seeds and Bio-PCR (bacteria cultured on agar media prior to PCR). In samples of 10 000 seeds containing one infected seed, *Cmm* could be detected only by Bio-PCR and in only one replicate out of five. In samples containing five or 10 infected seeds per 10 000 seeds, three of five and five of five replicates, respectively, were detected by the three detection methods. In samples of 5000 seeds, one infected seed could be detected in all five replicates only after adding a concentration step. A high correlation (R^2 = 0·9448) between artificially infested seeds and the disease incidence was found. Seed lots infested with less than 58 colony-forming units (CFU) per g did not cause disease under glasshouse conditions, whereas lots with about 1000 CFU g^{-1} caused disease in 78 plants out of 2000.

Keywords: bacterial canker, bacterial wilt, PCR, quarantine organism, seedborne disease

Introduction

Clavibacter michiganensis ssp. *michiganensis* (*Cmm*), the causal agent of bacterial canker of tomato, is a quarantine organism under the European Union Plant Health legislation (Anonymous, 1995) and in Israel according to the pest quarantine list of the Plant Protection Inspection Service. The organism causes bacterial wilt and canker of tomato (*Lycopersicon esculentum*) and is considered to be the most important bacterial disease of tomato, causing substantial economic losses worldwide (Strider, 1969; Davis *et al*., 1984). Infected seed is often considered to be the primary inoculum source (Thyr, 1969) and the major source for outbreaks of *Cmm* infection (Tsiantos, 1987). The population levels of the pathogen in/on the seed may be very low. Therefore, in addition to conventional detection methods, PCR-based procedures have been developed (Dreier *et al*., 1995; Santos *et al*., 1997). The pathogen is seedborne (Tsiantos, 1987) and even a low (0·01%) transmission rate from seed to seedling can initiate a serious epidemic in tomato-growing fields (Chang *et al*., 1991; Gitaitis *et al*., 1991). Therefore, indexing of tomato seed for the canker pathogen is an important step in disease control. Extensive efforts have been made to develop efficient and sensitive assays for detection of *Cmm* in commercial tomato seeds and seedlings (Gitaitis *et al*., 1991; Kritzman, 1991; Ghedini & Fiore, 1995; Fatmi & Schaad, 1988; Biggerstaff *et al*., 2000). The broad morphological variation among *Cmm* strains and the variable level of seed infection have increased the difficulty of developing a consistent detection method with uniform sensitivity and reproducibility. Although the commonest sample size used for detecting *Cmm* in tomato seeds is 10 000 (Bolkan *et al*., 1996), the seed companies, the International Seed Federations (ISF) and the International Seed Testing Association (ISTA) are still working on standardizing the procedures.

Studies of field production of transplants have shown that a single infected seed among 10 000 seeds can initiate an epidemic under favorable conditions (Chang *et al*., 1991; Gitaitis *et al*., 1991). However, there are no available published data on the correlation between field disease and the level of bacteria on/in seeds.

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The aims of the present study were to evaluate several seed extraction procedures used for detection of *Cmm* in seed lots and to determine the detection threshold of *Cmm* in relation to seed sample size and disease incidence under glasshouse conditions.

Materials and methods

Bacterial strains and different media

The *Cmm* strains used in this study are listed in Table 1. The strains were obtained from the collection in the Volcani Center and from A. Alvarez, of the University of Hawaii, USA. The bacteria were maintained on plates containing nutrient agar (NA; Difco, France) and incubated at 28°C for 48–72 h. Storage for short periods was carried out at 4°C and for long periods at –80°C in 40% glycerol.

The following media were used: SCM (Fatmi & Schaad, 1988); mSCM (Bolkan *et al*., 1996); D2 (Kado & Heskett, 1970) containing in addition 0·12 g L^{-1} cycloheximide; D_2ANX (Chun, 1982) containing (per L) 10 g glucose (dextrose), 4 g casein acid hydrolysate, 2 g yeast extract, 1 g NH_4Cl, 0·3 g $MgSO_4 \cdot 7H_2O$, 1·2 g trizma base, 15 g agar (Difco), 3 mg nalidixic acid, 100 mg cycloheximide and 13 mg polymixin B sulphate. The medium had pH 7·4 after autoclaving; CNS (Gross & Vidaver, 1979) without Daconil; mCNS containing (per L) 5 g peptone, 3 g yeast extract, 0·5 g KH_2PO_4, 2 g K_2HPO_4, 0·25 g $MgSO_4 \cdot 7H_2O$, 5 g LiCl, 15 g agar (Difco), 30 mg nalidixic acid, 50 mg cycloheximide and 38 mg polymixin B sulphate; YDC (Wilson *et al*., 1967); NBY (Vidaver, 1967); CBM is a modification of NBY medium containing (per L) 16 g nutrient broth, 4 g yeast extract, 4 g KH_2PO_4, 1 g K_2HPO_4, 0·246 g $MgSO_4 \cdot 7H_2O$, 5 g glucose, 15 g agar (Difco), 25 mg nalidixic acid, 32 mg polymyxin B sulphate, 0·2 g cycloheximide; PBS (phosphate buffer and saline) 0·1 M, containing (per L) 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.

For each medium, nonheat-labile components were dissolved in distilled water by steaming and then autoclaved for 15 min at 121°C. Heat-labile components were filter-sterilized using a FP 30/0,2 CA-2 filter (Schleicher & Schuell, Germany) and added to cooled, molten, autoclaved medium before pouring into 9 cm plastic Petri dishes. The plates were inoculated by spreading 100 μL of bacterial suspension and incubated at 28 ± 2°C for 4–11 days. For each medium and each dilution, three plates were used. Bacterial colonies that exhibited a typical appearance of *Cmm* on the various selective media, as compared with

Table 1 Percentage recovery of *Clavibacter michiganensis* ssp. *michiganensis* strains on different selective media

		Media[a]			PCR primer sets[b]	
Strain no.	Origin	CNS	D_2ANX	mSCM	CM_3 + CM_4	CMM-5 + CMM-6
4775	Michigan[d]	0	74[c]	139·6	+	+
4874	Gilroy CA[d]	17·9	46·5	32·3	–	+
4829	Ohio[d]	26·6	80	51·9	+	+
4748	Kenya (IPO)[d]	11·8	37·6	32·3	–	–
CA4866	Gilroy CA[d]	27·2	74·1	4·4	+	+
4598	WA state[d]	0	72·6	2	+	–
4791	China[d]	0	28·9	6·6	–	+
Fr	France	0	37·9	11·2	+	+
2701	N. Carolina[d]	2·2	27·8	1·5	+	+
IPO150	The Netherlands	0	7·9	1·2	–	+
4592	WA state[d]	0	100	0	+	+
Belg.	Belgium	0	100	0·1	+	+
NCPPB382	UK	0	100	100	+	+
4011	Ohio[d]	21·9	0	25	+	+
2645	California[d]	0	58	13	+	+
3699/2	Israel	4·8	16·2	13·7	+	+
IS34	Israel	100	100	60	+	+
79	Israel	12·2	61·1	100	+	+
104	Israel	71·4	40·0	85·7	+	+
110	Israel	49·1	10·4	89	+	+
112	Israel	34	15·7	100	+	+
116	Israel	6·1	19·6	72	+	+
120	Israel	46·9	52·5	100	+	+

Details of media are given in text.

[a]Bacterial cells were counted after growth of 11 days on CNS and mSCM, and after 6 days on D_2ANX. Data are mean numbers of cells.

[b]PCR tests were carried out as described in 'Materials and methods'. + indicates a positive response.

[c]Results are the percentage of CFU of bacterial cells recovered on each medium of the total cells grown on NBY medium for 3 days. For each medium and each dilution, two plates were used.

[d]Strains received from the collection of Ann Alvarez, University of Hawaii, USA. Other strains are from the collection in the Volcani Center.

reference strains, were further purified by streaking onto YDC medium, subjected to Gram staining (Lelliott & Stead, 1987), polymerase chain reaction (PCR) as described below, and enzyme-linked immunosorbent assay (ELISA) (Clark & Adams, 1977), kit no. 07063 (Loewe Biochemica GmbH, Germany).

To select the most suitable medium for isolation of *Cmm* from seeds, five *Cmm* strains [CA4866, Fr, IPO150, NCPPB382, IS34 (Table 1)] obtained from different countries were inoculated on seven different selective media (CNS, mCNS, SCM, mSCM, D_2ANX, D2, CBM) and their growth compared with that on the nonselective NBY agar. Three media (CNS, mSCM, D_2ANX) on which the morphology of the colonies was easily characterized, and at least two of which supported growth of the chosen strains, were selected for further testing of an additional 18 different strains (Table 1). Colony characterization was compared with that on YDC and SCM media, which are routinely used for determining colony morphology.

Infected and infested seed

Commercial tomato seed lots of cvs M82, 5656 and 870 were obtained from Hazera Genetics Ltd, Israel. Naturally infected seeds were extracted from ripe fruits of tomato plants (cv. 2526) showing typical disease symptoms. The seeds were not disinfected. The tissue containing the seeds and gelatinous material were removed from the fruits and incubated overnight at 25°C. The tissue was then washed in tap water with gloved hands to remove the gelatinous material and the seeds were dried in a laminar hood on a filter paper for 40 h.

The amount of *Cmm* in an individual seed was determined with 55 seeds. Each seed was chopped with a scalpel in a 1·7 mL Eppendorf tube. After adding 0·6 mL of PBS buffer, 100 μL of the extract was plated on each of three NBY agar plates.

Infested seeds were prepared as follows: a culture of *Cmm* strain IS34 grown on NBY for 48–72 h was suspended in sterile distilled water and adjusted to an optical density (OD) of 0·67 at 480 nm to result in 10^8 colony-forming units (CFU) per mL. Different concentrations of inoculum (10^2–10^5 cells mL^{-1}) were prepared by serial 10-fold dilution. Seed lots (50 g) were then infiltrated under a pressure of 1 atmosphere in 200 mL of the above dilutions of *Cmm* for 1 h. The infiltrated seeds were then dried on filter paper in a laminar-flow chamber for 2 days. The seeds were placed into a desiccator and kept for 3 weeks at room temperature. The infested seeds were used in the experiment conducted to determine the disease incidence. Three to four replicates from each of the contamination levels were used. The actual level of infestation of the seeds was determined by extracting 2000 seeds, using the procedures described below. To determine the resulting disease level, seeds were sown in the glasshouse and kept at 28°C, under 70–80% relative humidity and 12 h/12 h light/dark illumination. After 60 days, seedlings that expressed typical disease symptoms of *Cmm* (leaves wilting) were confirmed in the laboratory by extracting 0·2 g of leaf tissue in 1 mL saline with pestle and mortar. Tenfold serial dilutions were prepared in saline and 100 μL were plated on mSCM, D_2ANX and NBY media.

Extraction methods

Several extraction procedures were evaluated with samples of 2000 seeds (~7 g) from commercial lots. The procedures included grinding (G) with a mill; grinding and blending with a Stomacher laboratory blender (Model 400, Type BA 7021, Seward Medical, UK). (GS); soaking (S) in PBS buffer; and soaking and blending with a stomacher (SS). In procedures G and GS, dry seeds were ground in a Wiley mill, model 3383 (Arthur H. Thomas, USA) with a 20-mesh net, and then suspended in 35 mL of 0·1% (w/v) PBS agar. The suspension was shaken at 4°C for at least 16 h. To prevent cross-contamination between samples, the Wiley mill apparatus was disinfected by spraying 95% ethanol and flaming. In procedure GS, the suspension was blended for 2 min at normal speed (230 rpm) in a heavy-duty SM2-01 plastic bag (Interscience, France). In procedures S and SS, the seeds were suspended in 28 mL of sterile PBS supplemented with 0·1% agar at 4°C for at least 16 h; in the SS procedure the suspension was blended for 2 min in a stomacher. Serial 10-fold dilutions in saline were prepared and then transferred onto mSCM, D_2ANX and NBY media in dishes. After incubation at 28 ± 2°C for 4, 6 or 11 days on NBY, D_2ANX or mSCM, respectively, the number of suspected *Cmm* and other colonies growing on each Petri dish was recorded. Colonies suspected to be *Cmm* were purified by streaking onto YDC medium. All yellow colonies were subjected to Gram staining, ELISA and PCR as described below.

Samples of 5000 (~17 g) or 10 000 (~35 g) seeds were suspended in 70 or 140 mL, respectively, of sterile PBS supplemented with 0·1% agar (w/v). The samples were incubated at 4°C for at least 16 h, shaken for 2–3 h at room temperature, and then transferred to heavy-duty plastic bags and blended using a stomacher for 5 min at normal speed. Dilution and plating were carried out as described above. In addition, 10 mL of the suspension was centrifuged at 6000 g for 10 min; the pellet was resuspended in 1 mL of sterile PBS and 100 μL were added to three Petri dishes of each of the following media, mSCM, D_2ANX and NBY. For each experiment, positive control plates were also prepared, inoculated with *Cmm* reference strain NCPPB382.

To compare the four extraction methods, three lots, each of 2000 seeds, with differing infestation levels were used. The lots were prepared by mixing naturally infected seeds with seeds of cvs M82, 5656 and 870. No infected seeds were added to lot 1. Lots 2 and 3 contained five and 10 infected seeds, respectively. Colonies that developed on mSCM, D_2ANX, and NBY media were confirmed as *Cmm* by PCR and ELISA. The experiment was repeated three times with three replicates for each infestation level (total of nine replicates). Each replicate was tested on mSCM, D_2ANX, and NBY in three dilutions. The ratio

between the number of replicate agar dishes with and without *Cmm* colonies from a total of nine replicates was calculated for each extraction method.

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

The detection threshold was evaluated by adding different numbers of naturally infected seeds to noninfected seed samples of three different sizes: 2000, 5000 and 10 000 seeds. Extraction was done according to the sample size as described above. *Cmm* in each sample was detected by adding 100 μL onto each of CNS, mSCM, D_2ANX agar plates and by direct PCR from seed material and Bio-PCR which first cultured bacteria on agar prior to PCR.

Disease incidence determination

Three or four replicates of 2000 seeds artificially infested with bacteria (10^2–10^5 cells mL^{-1}) were sown in the glasshouse. After 60 days, the disease incidence was determined as the number of seedlings showing disease symptoms in each replicate of 2000 seeds. The presence of *Cmm* in the diseased seedlings was confirmed by PCR and agar plating as described above.

Pathogenicity tests

Pathogenicity tests on tomato seedlings were performed by puncturing the stem four times with a needle that had been dipped in a suspension of bacteria containing 10^7–10^8 cells mL^{-1}. The inoculum was prepared from a culture grown overnight on NA and suspended in saline. The plants were maintained at 28°C and symptoms were recorded after 7–21 days. For each strain, five plants were inoculated, and the test was conducted three times.

PCR conditions

PCR amplifications were carried out with primers CMM-5, CMM-6 (Dreier *et al*., 1995) and with CM_3 and CM_4 (Santos *et al*., 1997) in 25 μL volumes. The reaction mixture contained 1 mM $MgCl_2$, 0·2 μM primer (synthesized by Sigma-Aldrich Co., Israel), 1 U of *Taq* DNA polymerase (Super Nova, Madi Ltd, Israel), 50 μM each deoxynucleoside triphosphate (dNTP) (Boehringer, Germany) in 10 mM tris-HCl (pH 9·0), 50 mM KCl, and 0·1% Triton X-100, under two drops of mineral oil. For direct PCR, 10 μL of the seed extract were used as template DNA. For Bio-PCR, 100 μL of the seed extract were spread onto NBY medium and incubated at 28°C for 48 h. The culture was harvested by adding 1 mL of water and suspending the cells with glass-loop. A 10 μL aliquot of the bacterial suspension was added to the PCR reaction as template DNA. Amplifications were performed in a VDE0871/B thermal cycler (Cetus, Perkin Elmer, USA). Denaturation was done at 94°C for 5 min, followed by 40 cycles of 1 min at 94°C, 1 min at 55°C for CMM-5, CMM-6 or 62°C for CM_3, CM_4 and 30 s at 72°C; and a final extension for 5 min at 72°C. Strain NCPPB382 was used as a positive control in the PCR and water was used as a negative control. For direct PCR and Bio-PCR from seed extraction, the negative control consisted of samples without addition of infected seeds. The amplified products were subjected to electrophoresis in 1·2% agarose gel and stained with ethidium bromide.

Data analysis

The results were subjected to the CATMOD procedure and chi-squared test at $P \leq 0{\cdot}05$ to determine the statistical significance of the differences between measured parameters (SAS Institute Inc., version 8·02). Regression analysis was used to examine the disease incidence as a function of the seed infection level, with the aid of Microsoft Excel software, after the relevant data had been logarithmically transformed.

Results

Characterization and recovery of *Cmm* strains on different media

All the strains listed in Table 1 were identified as *Cmm* according to their appearance on semiselective media, Gram staining (+), and their reaction to the ELISA test. The morphology of the colonies was determined on YDC and SCM. All strains were yellow and mucoid on YDC except for strains 4748 and 2701, which were orange and dry or pale yellow, respectively. On SCM, strains 4755, 4829, Fr, 3699/2, IPO150, Belg, NCPPB382, 4011, 2645 and IS34 were mucoid to runny and grey in colour with internal black flecks; strains 4874, CA4866, 4598, 4791 and 4592 were mucoid, grey in colour; strain 4748 was dry and black; and strain 2701 was grey and not mucoid. Characterization by PCR showed that four strains (4874, 4748, 4791 and IPO150) did not react with the primers CM_3 + CM_4, and two strains (4748 and 4598) did not react with the primers CMM-5 + CMM-6. Pathogenicity tests that were carried out on tomato seedlings showed that 22 strains elicited typical disease symptoms with various degrees of pathogenicity. Strain 4748, which gave negative results in PCR reactions with the two sets of primers, was nonpathogenic but considered as *Cmm* because it reacted positively in the ELISA test. Since false positives and false negatives can still be found using PCR with these two sets of primers (Kaneshiro & Alvarez, 2001), it is necessary to employ more than one set of primers. All the pathogenic strains listed in Table 1 reacted with at least with one set of primers.

The recovery of *Cmm* strains on the three different media (CNS, mSCM, D_2ANX) was found to be highly variable (Table 1). For example, strain 4592 grew very well on D_2ANX but did not grow on CNS and mSCM, whereas strain 4011 did not grow on D_2ANX but grew on CNS and mSCM. Each of the tested strains grew on at least one of the selective media. On CNS the recovery of *Cmm* strains was in general the poorest; nine of the tested

strains did not grow. D_2ANX supported the growth of all the strains (except strain 4011) and the recovery of 14 strains was higher than on mSCM. The Israeli strains grew better on mSCM than on the two other media. These three media were subsequently used for comparisons of different extraction methods.

Infected and infested seeds

The amount of *Cmm* on individual naturally infected seed was determined with 55 seeds. It ranged between 156 and 3990 CFU per seed with an average of 935 ± 95. The amount of *Cmm* on artificially infested seed was determined for the different levels of inoculum. The ranges were 3–65, 76–115, 183–315 and 428–1013 CFU per g seeds for infestation level of 10^2, 10^3, 10^4 and 10^5 cells mL^{-1}, respectively.

Comparison of the effectiveness of four extraction methods for isolation of *Cmm* from tomato seeds

The extraction by grinding only, and grinding and stomacher (G and GS) resulted in a higher number of samples with *Cmm* than did the methods with soaking only and soaking with stomacher (S and SS) (Fig. 1). The addition of the stomacher step following grinding or soaking (GS, SS) did not increase the ratio compared with that obtained by grinding or soaking alone (G, S). Lot 1, which was used as control and was considered to be pathogen-free, contained a very low level of *Cmm* as shown by the extraction methods SS and G. Lot 2 did not differ significantly from lot 3 in any of the extraction methods.

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

The pathogen was detected in samples of 2000 seeds containing one infected seed, in all the five replicates and by all three detection methods (Table 2). In samples of 5000 seeds, one infected seed was detected in all five replicates only after adding a concentration step. In samples of 10 000 seeds, a positive result was obtained only by Bio-PCR, and in only one of five replicates. Two or five infected seeds could be detected in all five replicates of a 5000-seed sample, but in only three of five replicates of a 10 000-seed sample. In a 10 000 seed sample, 10 infected seeds were detected in all five replicates. Adding a centrifugation step to concentrate the extraction 10-fold improved the detection threshold only in samples of 5000 seeds.

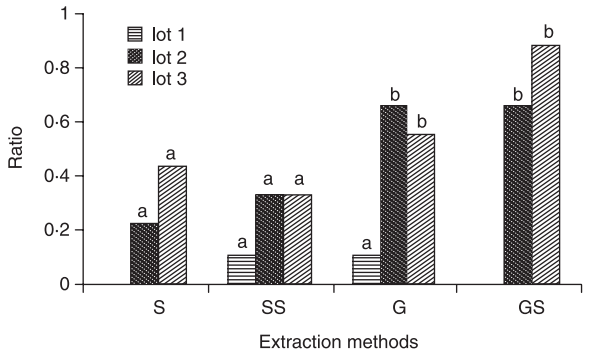

Figure 1 Ratio of replicate agar plates with positive colonies of *Clavibacter michiganensis* ssp. *michiganensis* from a total of nine inoculated replicate plates for each extraction method. The extraction methods were: S, soaking; SS, soaking and mixing in a stomacher; G, grinding; GS, grinding and mixing in a stomacher. Lot 1, control without addition of infected seeds; lot 2, addition of five infected seeds/2000; lot 3, addition of 10 infected seeds/2000. The test was performed in triplicate on three different media. Columns with different lower-case letters are significantly different ($P < 0{\cdot}05$).

Table 2 The detection threshold of *Clavibacter michiganensis* ssp. *michiganensis* in relation to sample size

	Detection method		
Infected seed[a]/sample	Plates[c] (–C +C[d])	Direct PCR	Bio-PCR
0/2000[b]	0/1[e]	0/1	0/1
1/2000	5/5	5/5	5/5
3/2000	5/5	5/5	5/5
0/5000[c]	0/5	0/5	0/5
1/5000	4/5 5/5	5/5	5/5
2/5000	5/5 5/5	5/5	5/5
5/5000	5/5 5/5	5/5	5/5
0/10 000	0/5 0/5	0/5	0/5
1/10 000	0/5 0/5	0/5	1/5
5/10 000	3/5 3/5	3/5	3/5
10/10 000	5/5 5/5	5/5	5/5
20/10 000	1/1 1/1	1/1	1/1

[a]Seeds were naturally infected.
[b]Seed extraction method with 2000 seeds: grinding and stomacher.
[c]Seed extraction method with 5000–10 000 seeds: soaking and stomacher.
[d]+C, –C with or without centrifugation step.
[e]The number of replicates with positive results (agar plates or PCR test) out of the total tested.

Disease incidence as a function of the seed contamination level

The germination percentages of all the contaminated seeds were above 96%. A high correlation ($r^2 = 0{\cdot}9448$) between seed contamination level and disease rating was found (Fig. 2). Seed lots infested with less than 58 CFU g^{-1} did not cause disease. Seed lots with about 1000 CFU g^{-1} resulted in 78 diseased seedlings out of 2000. *Cmm* was detected in all the diseased seedlings.

Discussion

Tomato seeds infected by *Cmm* act as sources of primary inoculum for bacterial canker epidemics (Gleason *et al*., 1993), and therefore efforts have been made to develop sensitive methods for detecting the pathogen in seeds (Fatmi & Schaad, 1988; Bolkan *et al*., 1996; Biggerstaff *et al*., 2000). Geng *et al*. (1983) identified two parameters needed to determine the reliability of a seed assay; the first

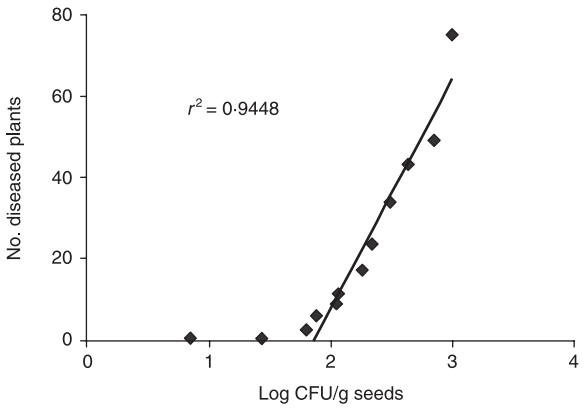

Figure 2 Incidence of bacterial canker in tomato seedlings as a function of level of seed infestation with *Clavibacter michiganensis* ssp. *michiganensis* measured as colony-forming units (CFU) per g of seed. Mean number of diseased seedlings per 2000 seeds from either three or four replicates.

is the probability of including at least one contaminated seed in the sample, and the second is the probability that assay sensitivity permits detection of the pathogen. For many seed lots there is the challenge of what could be termed the 'one in 10 000 problem' (Louws *et al*., 1999). Most sampling protocols cannot detect an infestation/infection at a threshold below one seed in 10 000, but such a threshold can still represent an economic risk (Louws *et al*., 1999). Field studies in the midwestern United States have shown that a single infected seed in 10 000 can initiate an epidemic under favourable conditions (Chang *et al*., 1991; Gitaitis *et al*., 1991). The SCM medium developed by Fatmi & Schaad (1988) is claimed to detect a single contaminated seed in samples of 10 000 seeds. However, seed-testing laboratories and industrial seed-screening programmes have not observed such sensitivity (G. Kritzman, ARO, Israel, personal communication), probably because of the variations in the levels of seed contamination and in the procedures used to extract the bacteria from the seeds. In the present study, it was shown that one infected seed in a sample of 10 000 could be detected only by Bio-PCR, and in only one replicate out of five. However, one infected seed among 5000 seeds can be detected in all the five subsamples by agar-plating after adding a concentration step, by direct PCR or by Bio-PCR (Table 2). Although the PCR-based methods are generally considered more sensitive than other detection methods (Louws *et al*., 1999), in this study, only Bio-PCR was more sensitive than dilution plating on semiselective media. These results could be explained by the low levels of saprophytic bacteria residing on the seeds in the specific seed lots used in this study. It is possible that in highly contaminated lots, the direct PCR methods would have an advantage over the other methods. It should be emphasized that the infected seed lot used in the present study had infection rates ranging from 156 to 3990 CFU per seed. In other experiments with artificially infested seeds with 5000–8000 CFU per seed, it was possible to detect one *Cmm* positive seed in 10 000 (results not shown). Because the natural level of *Cmm* in seeds is highly variable, the threshold should be set to detect the lowest level of infection.

Several semiselective media for isolating *Cmm* have been developed (Davis & Vidaver, 2001). Since different strains of *Cmm* may exhibit differing colony morphology on the same medium, and since no medium is foolproof, many seed-testing laboratories use several media simultaneously. In the present study, seven different semiselective media were examined with five well-characterized *Cmm* strains, originated from different geographical sources. Three media, CNS, D_2ANX and mSCM, were selected for further studies because they provided the best growth and recognition of most strains. Although the recovery of *Cmm* on CNS has been reported to be poor (Fatmi & Schaad, 1988), CNS was included since it was more inhibiting of other bacteria (Gross & Vidaver, 1979).

The sensitivity of any method for detection of bacteria in seeds depends on the extraction method (Roth, 1989). The present study demonstrated that the extraction of *Cmm* in seeds by grinding was more effective than by soaking. However, this result should be confirmed by testing several naturally infected seed lots from various sources. A process of grinding samples of 5000–10 000 seeds is not practicable due to technical difficulties and is limited to sample size of 2000 seeds, which represent small lots (10 kg) of very expensive seeds (e.g. hybrid seeds). The use of nondestructive assays for detecting *Cmm* in seed is still not satisfactory (Biggerstaff *et al*., 2000).

The potential of infected seeds to cause disease is a major concern and is economically important. A high correlation between the disease incidence, as measured under glasshouse conditions, and the level of bacteria added to seeds was found in the present study. This result has an economic impact since all tomato plants grown in Israel for seed production and for the fresh market (as opposed to processing production) are grown in glasshouses. The minimum infestation level required to cause disease in one plant was found when the contamination level was 58 CFU per g seed, whereas the highest tested infestation level of 1000 CFU g^{-1} elicited disease symptoms in 4% of the plants. It is not known whether the 58 CFU g^{-1} were isolated from one seed or several. However, when developing a risk analysis strategy for *Cmm* in tomato seeds, it should be considered that a very low level of bacteria on naturally infected seeds can cause disease. In the light of the available detection methods of *Cmm* in seeds, the results of the present study suggest that detection of one infected seed requires a test sample not larger than 5000 seeds.

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