Neth. J. Pl. Path. 99 (1993) 125–137

Detection of Clavibacter michiganensis ssp. michiganensis in tomato seeds by immunofluorescence microscopy and dilution plating

A.A.J.M. FRANKEN, G.C. KAMMINGA, W. SNIJDERS, P.S. VAN DER ZOUWEN and Y.E. BIRNBAUM

DLO Centre for Plant Breeding and Reproduction Research (CPRO-DLO), P.O. Box 16, 6700 AA Wageningen, the Netherlands

Accepted 2 March 1993

Abstract

A method for detecting *Clavibacter michiganensis* ssp. *michiganensis* in tomato seeds was evaluated. The method is based on rapid screening of tomato seed lots using indirect immunofluorescence staining (IF), followed by dilution plating of IF positive seed lots. Different polyclonal antisera, prepared against *C. michiganensis* ssp. *michiganensis* were tested for their specificity using IF. All strains of *C. michiganensis* ssp. *michiganensis* tested reacted with the polyclonal antisera. Two of nine saprophytic isolates from tomato seeds were positive with the antisera as well as with the control normal serum, but cells of these isolates were distinct in shape from cells of *C. michiganensis* ssp. *michiganensis*.

For extraction of the pathogen from the seed, seeds were either blended with a stomacher or soaked at 4–6 °C. The stomacher method yielded more fluorescent cells in IF than 24 h soaking of seed samples. However, soaking of seeds for 48 h generally yielded less saprophytes and overall higher numbers of *C. michiganensis* ssp. *michiganensis* colonies in dilution plating when compared to blending by a stomacher. SCM medium was generally more selective than KBT and modified CNS medium. However, the efficacy of the medium was dependent on the seed lot and/or extraction method used. Confirmation of suspected colonies with YDC (yeast-dextrose-carbonate medium), IF and a pathogenicity test on tomato seedlings proved to be highly reliable ($P > 0.95$). For routine testing of seed lots it is recommended to screen tomato seed lots after soaking seeds for 24 h at 4–6 °C with IF, followed by plating of IF-positive seed lots on modified CNS and SCM after soaking seeds for an additional 24 h.

Additional keywords: bacterium, detection, identification, media, pathogenicity-testing, serology, seed-borne.

Introduction

Clavibacter michiganensis ssp. *michiganensis* (Cmm) the causal agent of bacterial canker of tomato, is a seed-transmitted plant pathogenic bacterium (Bryan, 1930) under quarantine by the European Community (EC). One of the most practical ways to prevent the organism from spreading and establishing is the use of disease-free seeds.

Healthy seeds may be obtained by seed treatments, but seed treatments may not eradicate the pathogen in all instances or may affect seed germination (Dhanvantari, 1989). Treated seeds should always be tested to determine the effectiveness of the treatment (Fatmi et al., 1991). Therefore, sensitive and reliable detection methods are needed, which can be used in routine indexing programs. Several detection methods have been described,

previously. Van Vaerenbergh and Chauveau (1987) developed a tomato seedling test to confirm IF tests. Seeds were vigorously shaken in phosphate buffered saline with cycloheximide at 25 °C and extracts were checked for presence of coryneform fluorescent cells after 24 and 72 h. Extracts of IF-suspected seed lots were injected in tomato seedlings. After incubation tomato seedlings were checked for symptom development and presence of the pathogen. Fatmi and Schaad (1988) developed a semi-selective medium (SCM) for isolation of Cmm from tomato seeds, using a lab blender (stomacher). Other media which have been used include KBT(S) medium (Dhanvantari, 1987; Gitaitis et al.; 1989), or modifications of CNS medium (Dhanvantari, 1989; Gitaitis et al., 1989; Gitaitis, 1990; Chang et al., 1991; Gitaitis et al., 1991).

The method of Fatmi and Schaad (1988) is available but requires 10–12 days or longer, certainly. For routine indexing of tomato seed lots quick, sensitive and specific methods are needed. A major advantage of IF is speed and high sensitivity (Schaad, 1982). However, dead and viable cells can not be distinguished. The advantages of plating are the high specificity and the recovery of viable cultures. In the present study, we describe our research with respect to the development of a detection method based on rapid screening of seed lots with IF, followed by confirmation of IF-positive seed lots by dilution plating.

Materials and methods

Extraction of Clavibacter michiganensis ssp. *michiganensis.* The seed lots, infected with Cmm were naturally contaminated and originated from several parts of the world (Europe, Africa, USA and Asia). All seed samples (obtained from different seed companies) were harvested between 1979 and 1990, and stored at 4–6 °C. Each subsample of a seed lot contained 15 g of seed (approximately 6250 seeds). For each extraction method, 150 ml sterile phosphate Tween buffer, containing per liter 7.75 g of Na_2HPO_4, 1.65 g KH_2PO_4 , 0.2 ml of Tween 20 (Fatmi and Schaad, 1988) and 100 mg of cycloheximide, was added to the subsample. For the soaking procedures, seeds were soaked for 24 h, 48 h or 72 h at 4 °C, shaken for 1 min and centrifuged, as described below. For blending seeds, the procedure described by Fatmi and Schaad (1988) was slightly modified. In short, seeds were first incubated for 15 min at 4 °C (H. Bolkan, unpublished). Then blended in a stomacher for 15 min at room temperature and centrifuged (see below). For soaking, IF was used as a first screening after 24 h soaking, and samples were plated after 48 h soaking. For blending with the stomacher, IF and dilution plating were both used for the same extract at the same time.

Extraction under vacuum was done as reported by Franken and Van der Zouwen (1988). Seeds, in the same ratio of buffer to seed as mentioned above, were put in a desiccator. Air was removed with a vacuum pressure pump (to 2.2 mbar) for 5 min. Afterwards, the desiccator was shut off from the ambient air for a specified period of time. After this period, the desiccator was opened and seeds were vigorously shaken by hand for 1 min. When varying the duration of the vacuum extraction, each extraction was done on a separate set of replications. For all extraction methods, the suspensions were stored for 10 min at room temperature (24 °C ± 1.5 °C) to settle down the seed debris after shaking for 1 min. For all extraction methods, 50 ml of each seed washing was removed by decanting the solution over one layer of sterile cheese cloth. Subsequently the 50 ml of seed washing was centrifuged for 10 minutes at 12 000 g_n at 4 °C. Each pellet was suspended in 1 ml of sterile phosphate buffer without Tween 20 and the suspension stored on ice for 2 h. Tenfold dilutions were made from this suspension and used for plating (0, 10^{-1}, 10^{-2} dilution) and IF (0, 10^{-1} dilution).

Isolation of Clavibacter michiganensis ssp. *michiganensis*. Fifty microliter of the non-diluted, 10^{-1} and 10^{-2} dilutions were plated onto duplicate plates of SCM (Fatmi and Schaad, 1988) and CNS (Gross and Vidaver, 1979) modified by the omission of lithium chloride and Bravo 6F and increasing the cycloheximide concentration to 200 mg/l (mCNS; Gitaitis, 1990). In initial experiments, the KBT medium was also used (King's medium B with potassium tellurite and nalidixic acid and cycloheximide; Dhanvantari (1987)).

All plates were placed in the refrigerator and used 2–7 days after preparation. Inoculated plates were incubated at 27 °C and colonies counted after 5–14 days. Reference strain 542 of Cmm was plated as control (Table 1).

Identification of suspected colonies. When present, a minimum of five Cmm suspected colonies and at least two non-typical colonies per 15 g seed sample were transferred from

Table 1. Bacterial strains used for specificity testing of polyclonal antisera, prepared against *Clavibacter michiganensis* ssp. *michiganensis*.

Bacteria	Strain designation	Source[a]
Clavibacter michiganensis ssp. *michiganensis*	63, 64, 324	1
	183, 255, 630, A7, B8, 542R	2
	500, 501, 541, 542, 543, 544, 545	3
	84-69-4, 84-35-4, 14-75, 85-5-1	4
	886, 1397, 2323, 3226, 3239, 3341, 2034	5
	B1	6
	2551-69, 2544-69, 2307-60, 1437-86, 2545-69	7
Clavibacter michiganensis ssp. *insidiosus*	533	3
Curtobacterium flaccumfaciens ssp. *flaccumfaciens*	547	3
Pseudomonas aeruginosa	60	3
Pseudomonas syringae pv. *lachrymans*	520	3
Pseudomonas syringae pv. *maculicola*	154	3
Pseudomonas syringae pv. *phaseolicola*	111, 570	3
Pseudomonas syringae pv. *pisi*	518, 519	3
Pseudomonas syringae pv. *syringae*	126, 1147	3, 4
Pseudomonas viridiflava	540	3
Xanthomonas campestris pv. *amoraciae*	373	3
Xanthomonas campestris pv. *campestris*	367	3
Xanthomonas campestris pv. *carotae*	–	8
Xanthomonas campestris pv. *phaseoli*	375	3
Xanthomonas campestris pv. *phaseoli* (var. fuscans)	382, 762	3
Xanthomonas campestris pv. *translucens*	B1117	9
Xanthomonas campestris pv. *vesicatoria*	512, 523	3
Xanthomonas maltophilia	1974	5
Unidentified saprophytes	236, 870, 254, 457, 937, 951, 959, NA 5, NA 11	2

[a] Source names and location: 1 = Plant Protection Service (PD), the Netherlands; 2 = Authors; 3 = Research Institute for Plant Protection (IPO-DLO), the Netherlands; 4 = B. Rat, France; 5 = National Collection of Plant Pathogenic Bacteria, England; 6 = C. Mortensen, Denmark; 7 = International Collection of Micro-organisms from Plants, New Zealand; 8 = T.-L. Kuan, USA; 9 = N.W. Schaad, USA.

mCNS, SCM or KBT to yeast-dextrose-carbonate medium (YDC; Schaad, 1989) for obtaining single colonies with typical morphology of Cmm. The YDC plates containing the bacterial colonies were incubated for 2 days at 27 °C. At the same time, all selected colonies were also tested with IF. For the pathogenicity test, minimally two suspected Cmm colonies (YDC-positive and IF-positive; YDC-positive colonies are yellow and mucoid) and two non-typical colonies per subsample were used. Reference strain 542 was used as control (Table 1).

Pathogenicity test. All isolates to be tested were grown on YDC for 48 h and then suspended in sterile phosphate buffer without Tween and cycloheximide to give approximately 10^7 colony-forming units (cfu) ml^{-1} of bacteria, as determined by dilution plating on tryptone soya agar (Oxoid). Tomato seedlings at the second true leaf stage were inoculated by cutting the first leaf and introducing the bacterial suspension at the wounds according to Moffett et al. (1983). Inoculated tomato plants were grown in the greenhouse at 25 °C with 16 h light and 8 h darkness and regularly inspected for canker and wilting symptoms for 21 days. Plants under the same conditions but inoculated with sterile phosphate buffer served as controls.

Seed disinfection. Infected tomato seeds were soaked in 0.6% sodium hypochlorite (NaOCl) for 15 min or 0.6 M hydrogen chloride (HCl) for one hour (Dhanvantari, 1989). Fifty per cent of the treated seeds were rinsed by shaking them vigorously in running tap water for 15 min. Both, the rinsed and unrinsed seeds were placed separately on filter paper to dry overnight at room temperature (24 °C ± 1.5 °C). In some experiments, 0.2% sodium thiosulfate was added to the extraction buffer to eliminate the effects of residual chlorine after NaOCl treatment (Maddox and Hubbard, 1990).

Antisera and immunofluorescence microscopy (IF). Polyclonal antisera were prepared in rabbits against whole living cells at the Research Institute for Plant Protection (IPO-DLO), Wageningen, the Netherlands (Vruggink and Maas Geesteranus, 1975). Indirect IF (Van Vuurde et al., 1983) was used for specificity testing of polyclonal antisera with pure cultures of pathogens and saprophytes (Table 1). For testing seed lots with indirect IF, 50 µl of the non-diluted and 10^{-1} dilution was dried and fixed as described by Van Vuurde et al. (1983) onto a 8 mm well of a 10 wells microscope slide. For analysis of the seed extract the next procedure was followed: 20 microscope fields were examined if there was at least one positive cell in one field on average, if there were less cells than 20 cells per 20 microscope fields (field coefficient = 18, objective magnification ×63, internal magnification ×1.25, ocular magnification ×10), at least 100 microscope fields were checked, and when no cells were found in 100 fields the whole well was examined. Presence of 'typical' claviform (club-shaped) or dividing cells (Fig. 1A, 1B) increases the probability of recognizing cells of Cmm from most saprophytic bacteria and thus improves the interpretation of IF-slides (Rat, personal communication).

Simultaneously prepared slides, stained with antiserum against *Pseudomonas syringae* pv. *phaseolicola* and slides with Cmm stained with antiserum against Cmm served as negative and positive controls, respectively.

Statistical analysis. All data were converted to either cells or colony forming units (cfu) per ml of non-concentrated seed extracts. For statistical processing, logarithms to base 10 (log) of the cells of cfu were calculated. To include all zero readings of the plates and IF-slides, 1 cell or 1 cfu was added to all data. Analysis of variance was done with the statistical program Genstat 5 (Numerical algorithms Group Ltd, Oxford, United Kingdom).

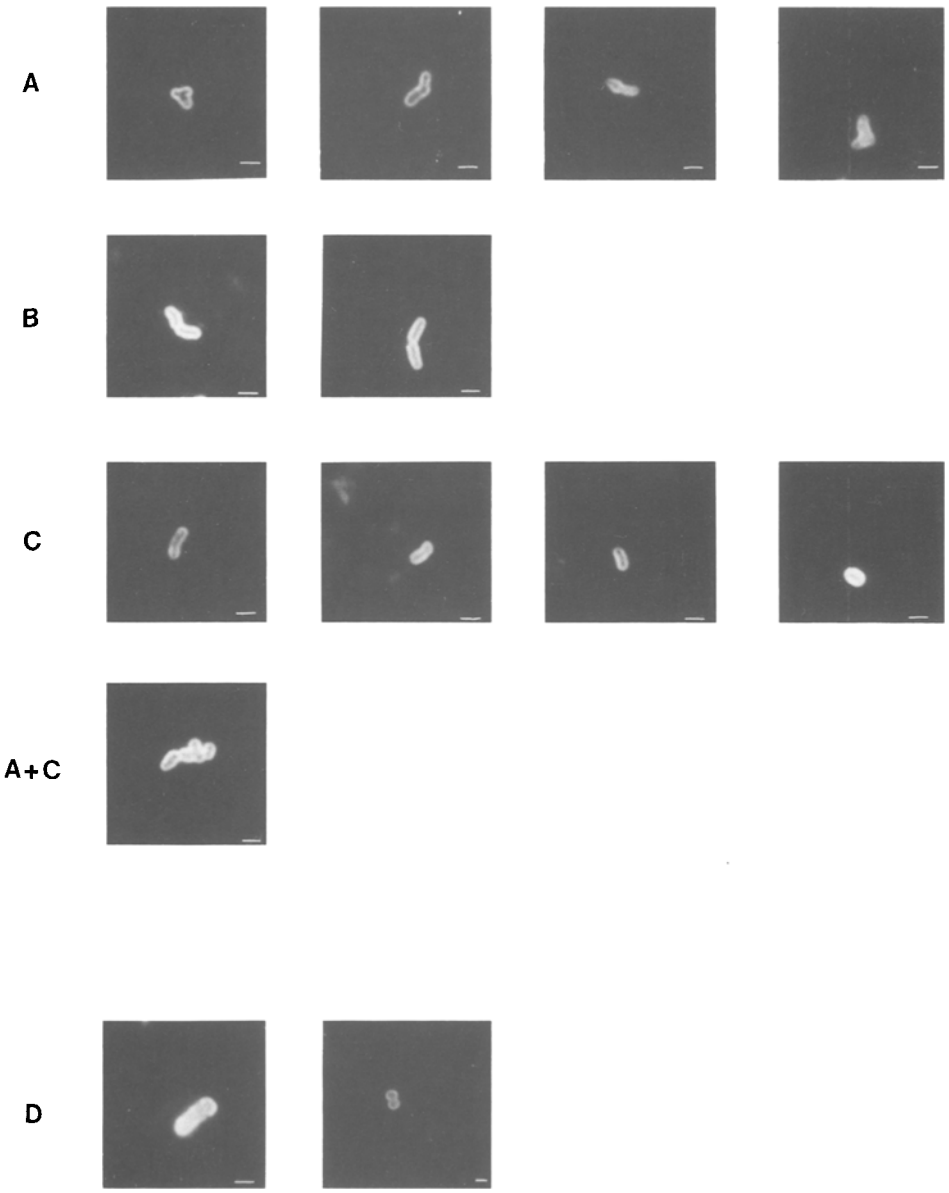

Fig. 1. Scheme of different cell types of *Clavibacter michiganensis* ssp. *michiganensis* in immunofluorescence microscopy. Each white bar represents 1 μm. A = 'typical' *C. michiganensis* ssp. *michiganensis* cells, often claviform-shape; B = dividing cells of *C. michiganensis* ssp. *michiganensis*; C = cells of *C. michiganensis* ssp. *michiganensis* not clearly dividing or 'typical'; A + C = a cluster of different cell types; D = cross-reacting bacteria with at the right, cells similar to cells of isolates NA 5 and NA 11.

For calculation of ratios of cfu of Cmm relative to the total number of cfu isolated, plates without any colonies of Cmm were included.

Results

Specificity of the antisera. In preliminary experiments, ten polyclonal antisera were tested separately against ten strains of Cmm, one strain of *C. michiganensis* ssp. *insidiosus*, one strain of *Curtobacterium flaccumfaciens* ssp. *flaccumfaciens*, six pseudomonads, six xanthomonads and one saprophyte isolated from tomato seed (Table 1). In all instances, no cross-reaction was observed with the pseudomonads, the xanthomonads, or the saprophyte tested. *C. michiganensis* ssp. *insidiosus* cross-reacted at a dilution factor of 900, which is the optimum working dilution (the highest dilution producing a strong discernible antigen–antibody reaction) of some strains of Cmm. Polyclonal antisera differed in optimum working dilutions only. Three antisera with lower optimum working dilution factors (900) and three antisera with higher optimum working dilution factors (2700) were mixed in equal volume amounts and further evaluated with 33 strains of Cmm, one strain of *C. michiganensis* ssp. *insidiosus*, eight pseudomonads, eight xanthomonads, and saprophytes (Table 1). Except for differences in the optimum working dilutions little differences were found between the two mixed and the individual antisera tested. All strains of Cmm and the single strain of *C. michiganensis* ssp. *insidiosus* were positive at antisera dilutions of 1:300–1:2700. One strain of Cmm (2544-69) gave a weak reaction at a dilution factor of 300 and a strong reaction at a dilution factor of 100 with the antisera. None of the pseudomonads and the xanthomonads reacted at any of the dilutions tested. Two isolates of saprophytic bacteria (NA 5 and NA 11) were positive (Fig. 1D). However, they were easily distinguished from Cmm by their cell morphology and fainter staining with dilution factors of 100 and 300; at higher dilutions (900, 2700, 8100), they did not react. NA 5 and NA 11 were also positive with the polyclonal normal serum as well as the control antiserum at dilution factor 100. When testing seed lots, the mixed antiserum with relatively high optimum working dilutions (Tables 3 and 4) or one of the individual antisera with relatively high optimum working dilution (Table 2) were used at a dilution factor of 100. In seed washings, large yeast cells as well as cells similar in morphology to NA 5 and NA 11 gave a reaction with the antisera. Cells of Cmm (Fig. 1A, B and C), however, were clearly distinct in morphology from these cross-reacting cells (Fig. 1D). The appearance of 'typical' and dividing cells (Fig. 1A and 1B) depended on the seed lot and extraction method used ($P < 0.001$). Generally, these cells constitute ca. 11% (mean percentage for the stomacher method) to c. 21% (mean percentage for the 24 h soaking procedure) of the total amount of suspected fluorescent cells. The cells that are not clearly typical or dividing (Fig. 1C) were most abundantly present in the samples tested.

Extraction methods. The efficacy of three different extraction methods for recovering Cmm from tomato seeds were first compared for two seed lots using KBT, SCM and mCNS. Table 2 shows that the vacuum extraction method generally resulted in the lowest number of cfu of Cmm in comparison to the stomacher method and soaking method. In IF, differences in recovery of Cmm between vacuum extraction and the other extraction methods were depending on the sample tested and duration of the vacuum extraction method ($P = 0.017$). A 5 minute vacuum extraction yielded similar number of cells as the stomacher method, whereas 120 min vacuum extraction yielded less cfu than the stomacher method. In dilution plating, differences found between the vacuum extraction periods were also depending on the medium and seed lot tested ($P < 0.001$). Longer soaking times

Table 2. Efficacy of three extraction methods for detection of Clavibacter michiganensis ssp. michiganensis in tomato seeds with dilution plating and immunofluorescence microscopy (IF).

Extraction method	Detection assay							
	dilution plating[1] (log cfu/ml of *C. michiganensis* ssp. *michiganensis*)						IF[2] (log fluorescent cells/ml)	
	seed lot	B187			383			B187	383
	medium	KBT	mCNS	SCM	KBT	mCNS	SCM		
Vacuum extraction									
5 min		0.00[a]	0.00	0.00	0.43	0.45	0.76	2.14	2.83
15 min		0.00	0.00	0.03	0.37	0.29	0.40	1.35	2.40
30 min		0.00	0.00	0.00	0.46	0.44	0.54	1.46	2.84
60 min		0.00	0.03	0.00	0.00	0.52	0.63	2.06	2.59
120 min		0.03	0.00	0.07	0.00	0.95	0.89	1.87	1.35
Stomacher		1.24	1.06	0.91	2.38	1.59	2.25	2.65	2.72
Soaking									
24 h		0.43	0.12	0.07	1.84	0.94	1.80	0.99	2.25
48 h		0.65	0.50	0.64	2.43	1.89	2.55	1.73	2.34
72 h		0.00	0.81	0.94	2.54	1.94	2.66	2.09	2.45

[1] The number of replications for dilution plating: $n = 4$ for vacuum extraction, $n = 2$ for stomacher and soaking; standard errors of differences of means is 0.237 for comparison of means with minimum replication ($n = 2$), and 0.190 for comparison of a mean with minimum replication and one with maximum replication ($n = 2$ with $n = 4$), and 0.155 for comparison of means with maximum replication ($n = 4$).

[2] The number of replications for IF is 6; standard error of differences of means is 0.393.

[a] This treatment was positive at a very low level (log cfu per ml was 0.001).

A specificity-tested polyclonal antiserum was used at a 1:300 dilution in IF (for details, see text).

generally increased the number of colonies found in dilution plating, except for plating of seed extract of lot B187 on KBT medium. In IF more cells were found with longer soaking times. Seed lot B187 generally yielded less colonies on the plates and less cells in IF ($P = 0.002$) than seed lot 383.

As a result of these experiments, the vacuum extraction procedure was omitted in subsequent experiments because isolation of Cmm was generally less successful with this method. The efficacy of the extraction method used in dilution plating partly depended on the seed lot tested ($P < 0.001$) (Table 3). Whereas, the stomacher method yielded more colonies in extracts of seed lot B212, the contrary was found in extracts of seed lots B159, B210 and B222. Overall, the soaking method yielded more colonies on SCM and mCNS than the stomacher method ($P = 0.006$, as determined by a sign test on data in Table 3 for SCM and mCNS). In contrast, the stomacher method yielded more fluorescent cells than the soaking method in IF. The non-specific background staining, however, was much stronger when using the stomacher method. This is attributed to the presence of seed debris. Also IF-slides were easier to read when using the soaking procedure.

Media. In initial experiments (Table 2) the KBT medium was also used. On this medium Cmm colonies appeared 2–5 days earlier than on mCNS or on SCM medium. However,

Table 3. Dilution plating on mCNS and SCM, and immunofluorescence microscopy (IF) for detecting Clavibacter michiganensis ssp. michiganensis in tomato seeds using two extraction methods.

Seed lot	Dilution plating[1] (log cfu per ml of *C. michiganensis* ssp. *michiganensis*)					IF[2] (log fluorescent cells per ml)	
	Extraction method	Stomacher		Soaking (48 h)		Stomacher	Soaking (24 h)
	Medium	mCNS	SCM	mCNS	SCM		
B159		0.00[1]	0.00	0.06	0.31	1.36[2]	0.91
B210		0.13	0.10	0.22	0.35	0.36	0.10
B212		0.37	0.24	0.00	0.07	*	1.74
B220		0.00	0.00	0.00	0.00	0.00	0.00
B221		0.00	0.50	0.21	0.78	2.47	1.82
B222		0.57	0.14	1.00	0.91	3.93	3.40
B223		0.40	0.03	0.62	0.29	4.00	3.50
B235		*	*	0.00	0.00	*	0.07
B239		0.00	0.00	0.00	0.90	1.37	0.54
B382		0.00	0.00	0.00	0.00	0.73	0.35
B826		*	*	0.10	0.32	*	4.48

[1] The standard error of differences of means is 0.158 for comparison of means with maximum replications ($n = 4$, all seed lots except B212), 0.193 for comparison of a mean with minimum replication and one with maximum replication ($n = 2$ with $n = 4$) and 0.223 for comparison of means with minimum replications (n=2; B212 only).

[2] The standard error of differences of means is 0.111 for comparison of means with maximum replication ($n = 4$, all seed lots except B212), 0.136 for comparison of a mean with minimum replication and one with maximum replication ($n = 2$ with $n = 4$) and 0.157 for comparison of means with minimum replications ($n = 2$; B212 only).

* = not tested. A mixture of three specificity-tested polyclonal antisera was used at a 1:300 dilution in IF(for details, see text).

due to the presence of large numbers of saprophytes suspected colonies were more difficult to find than on the mCNS and SCM medium. The average ratio of cfu of Cmm relative to the total number of cfu found on the plates was 5.1 ± 10.0%, 14.9 ± 16.0%, and 20.5 ± 21.7% for KBT, mCNS and SCM (n = 56 for each medium), respectively. Therefore, in subsequent experiments (Table 3), KBT was not used. Table 3 shows that the efficacy of the media (the log cfu of Cmm) depended on the extraction method used ($P = 0.014$) or seed lot tested ($P < 0.001$). The average ratio of cfu of Cmm relative to the total number of cfu found on the plates was about equal for mCNS and SCM (1.8 ± 5.4% and 1.7 ± 5.5%, respectively ($n = 38$)) for the stomacher method. These ratios were much higher for the soaking method, viz. 8.7 ± 18.4% and 13.9 ± 21.5% for mCNS and SCM, respectively ($n = 46$).

Confirmation of suspected colonies from mCNS and SCM. Of the 1039 colonies selected at random from SCM and mCNS plates 91.0% ($n = 612$) and 91.3% ($n = 427$) were recognized as positive on YDC medium, respectively. All YDC-negative colonies ($n = 195$ for mCNS and $n = 51$ for SCM) did not react in IF, but 94.6% (mCNS) and 98% (SCM) of the YDC-positive colonies were positive in IF. All of the both YDC-positive and the IF-

Table 4. Effect of seed disinfection agents on the detection of *Clavibacter michiganensis* ssp. *michiganensis* in tomato seeds with dilution plating and immunofluorescence microscopy (IF).

Seed lot	Treatment	Rinsing with water	Dilution plating[1]				IF[1]	
			Stomacher		Soaking (48 h)		Stomacher	Soaking (24 h)
			mCNS	SCM	mCNS	SCM		
B223	control	+	0.00 (± 0.00)	0.19 (± 0.28)	0.00 (± 0.00)	0.00 (± 0.00)	2.70 (± 0.11)	1.54 (± 0.04)
		–	0.27 (± 0.66)	0.00 (± 0.00)	0.55 (± 0.37)	0.00 (± 0.00)	4.00 (± 0.19)	3.51 (± 0.05)
	NaOCl	+	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	3.26 (± 0.01)	0.35 (± 0.49)
		–	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	3.66 (± 0.24)	1.40 (± 0.00)
	HCl	+	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	3.51 (± 0.12)	0.35 (± 0.49)
		–	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	3.26 (± 0.26)	0.83 (± 0.18)
B221	control	+	0.00 (± 0.00)	1.43 (± 0.43)	0.17 (± 0.33)	0.11 (± 0.26)	0.63 (± 0.89)	0.00 (± 0.00)
		–	0.00 (± 0.00)	0.59 (± 0.58)	0.10 (± 0.16)	0.47 (± 0.40)	2.10 (± 0.84)	0.61 (± 0.90)
	NaOCl	+	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.53 (± 0.74)	0.00 (± 0.00)
		–	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.00 (± 0.00)	0.84 (± 0.95)	0.17 (± 0.47)

[1] Log cfu per ml of *Clavibacter michiganensis* ssp. *michiganensis* in dilution plating, log fluorescent cells per ml in IF. Controls (seed lot not rinsed with water and not treated with NaOCl or HCl) of B223 and B221 were tested in six and eight replications, respectively. Other combinations were tested in two and four replications for seed lot B223 and B221, respectively. A mixture of three specificity-tested polyclonal antisera was used at a 1:300 dilution (for details, see text).

positive colonies tested were pathogenic to tomato (n = 303) and the both YDC-negative and IF-negative colonies (n = 25) tested were non-pathogenic to tomato. None of the YDC-positive colonies were negative in IF and positive in the pathogenicity test.

Influence of seed disinfection agents in dilution plating and IF. Seed lot B223 was treated with NaOCl and HCl. B221 was treated with NaOCl only. No colonies were found in dilution plating after the treatments regardless whether seeds were rinsed with water following the treatment or not (Table 4). Rinsing of seeds that had not been treated chemically, usually reduced the number of cfu found on the plates. In IF, however, in all instances fluorescent cells were found after the treatments (Table 4). Also in IF, rinsing of seeds with water considerably reduced the number of fluorescent cells observed. Seed treatments also reduced the number of fluorescent cells found, especially with the soaking procedure (Table 4).

Discussion

Our polyclonal antisera showed a high degree of specificity in IF. For testing tomato seed lots with IF, the presence of 'typical' and dividing cells (Fig. 1) gives extra confidence that cells are actually Cmm. However, the method is not 100% specific; reactions with saprophytes were also found. The number of cross-reactions is largely depended on the extraction method used. When using high temperatures and strong agitation, cross-reacting saprophytes are more prevalent. Soaking at low temperatures without shaking of seeds as used in this study strongly restricts the multiplication and release of these saprophytes. Although the cells of the saprophytes were clearly different from Cmm cells, they may lead to some difficulties in the reading of the IF slides by unexperienced workers. The significance of different cell types in the epidemiology of the pathogen is not clear yet. For seed testing, we consider every positive cell (at an optimum working dilution of the antiserum) of a similar shape and size to control slides of Cmm to be positive.

Our study showed that the stomacher and the soaking method resulted in similar recoveries of Cmm. In the soaking method presented here, we have not shaken the seed. Fatmi and Schaad (1988) have shown that blending tomato seed by a stomacher for 15 min significantly results in more cfu of Cmm than washing for 72 h at 2 °C by shaking seeds in a flask. Shaking seeds usually stimulates the release of interfering saprophytes and seed debris (Roth, 1989). This could affect the sensitivity of the assay. Blending results in the rapid and strong release of cells from the in- and external of the seed, but also in the release many saprophytes and antibacterial substances. The soaking procedure (without shaking) is less vigorous, cells are probably first released from the external and within 24 h (full imbibition of the seed takes maximally 6 h, H.L. Kraak, personal communication) from the internal of the seed. This procedure yielded fewer cells in IF than the stomacher method, but also fewer saprophytes indicated by the higher pathogen to saprophyte ratios on the media. Therefore, the soaking method without shaking as discussed in this study, gave similar or higher recoveries of Cmm than the stomacher method. Using soaking, followed by blending with the stomacher resulted in more cells in IF than when blending alone (A.A.J.M. Franken, unpublished). In dilution plating no differences were found between 24 h and 48 h soaking and blending with the stomacher before or after soaking with respect to log cfu of Cmm. The vacuum extraction method yielded lower numbers of saprophytes (Franken and Van der Zouwen, 1988), but also lower numbers of Cmm colonies.

It was shown that efficacy of the medium was dependent on the seed lot and the extraction method used. This is very likely due to the level and composition of the microflora

in/on the seed. Our results agree with Gitaitis (1990) that at least two media should be used for tomato seed testing. We recommend to use both mCNS and SCM. SCM has been improved recently, basically by replacing sucrose by mannose and omitting Chapman tellurite (Waters and Bolkan, 1992). However, we have shown that SCM sufficed well to isolate Cmm with the soaking procedure. Therefore, we did not use the modified medium. Confirmation of suspected colonies by growth on YDC, IF, and pathogenicity test on tomato seedlings proved to be highly reliable when used in combination for identification of Cmm. Only a small percentage of YDC-positive colonies was negative in pathogenicity testing and IF. These colonies were saprophytic isolates which were erroneously identified as Cmm on the basis of the colour of the colony on YDC. However, the colony morphology was different (less mucous).

After disinfection of seed lots fluorescent cells were still present in IF. These cells may be non-viable as indicated by the dilution plating results, or IF may detect (viable) cells below the detection threshold of dilution plating. We have looked into the possibility whether the detection assay may also have been disturbed by residual HCl or NaOCl in the extraction buffer. No pH changes were measured in the extraction buffers when comparing treated and untreated seeds. Rinsing treated seeds with water very likely washes away most of the residues of the chemicals. The decrease in number of fluorescent cells after rinsing untreated seeds indicates that the contamination by Cmm was in part superficial. Addition of sodium thiosulfate to reduce residual effects of chlorine as reported by Maddox and Hubbard (1990), did not have any significant positive effects on the recovery of the pathogen, but increased the recovery of saprophytes (not shown). This indicates a detoxification activity of thiosulfate for some bacteria. It should be emphasized that we have not studied the effect of NaOCl or HCl on the recovery of Cmm by plating seeds directly onto SCM, as was used by Fatmi et al. (1991). Although these tests may give more accurate information on the efficacy of seed treatments in plating assays, they will only work when seeds are free of saprophytes. Moreover, they are time-consuming and, therefore, not recommended for routine assays (Fatmi and Schaad, 1989). The seed-extract, agar-plating assay as described in this study can generally be used to obtain rough data on the efficacy of seed treatments. When using both IF and plating for evaluating seed treatments, the risk of false-negative results is low (Table 4).

Although we have not found clear indications of interference of HCl and NaOCl with the detection assay, we suggest to always test first untreated seed lots to avoid possible interference of chemicals with the assay.

Our results of screening seed lots with IF after 24 h soaking, followed by plating of IF-positive seed lots on mCNS and SCM after 48 h agrees with previous results found for *Pseudomonas syringae* pv. *phaseolicola* (Van Vuurde and Van den Bovenkamp, 1987). Up until now, no both IF-negative results at 24 h and dilution-plating positive results at 48 h were obtained, indicating that IF is indeed suited for use as a screening technique. The advantage of the method proposed here is that the IF results are confirmed by plating. This is more reliable than using IF as a sole test and faster than using IF in combination with a plant test (Van Vaerenbergh and Chauveau, 1987). The advantage of including IF in a routine indexing program is that it saves the costs and labour of dilution plating if seed samples are found negative in IF. If seed samples are negative in IF, a seed health test result is available within 24–48 h. Examination of one well of a multitest slide in IF, representing one seed sample of 10 000 seeds, usually does not take more than 5–15 min, if fluorescent cells are found in 20–100 microscope fields (A.A.J.M. Franken, unpublished). If seed samples are negative in IF, it takes usually 10–20 min to check the complete well of an IF-slide. It should be emphasized, however, that one needs to have some experience in reading of IF-slides to validate the cell types. Recently, it was demonstrated in the

Netherlands that reproducible results were obtained between experienced laboratories and unexperienced laboratories but intense training was required before these comparative tests were organized (A.A.J.M. Franken, unpublished). A comparative test within the International Seed Testing Association (ISTA) proved that counting of the cell types (Fig. 1A, B, C and D) in IF-slides can be well standardized (ISTA, 1988). However, considerable disagreement was present with respect to the validation of the results. Therefore, we recommend that a positive IF result should be confirmed by dilution plating. The dilution-plating method used after 48 h soaking of seeds enables to isolate viable bacteria and to confirm IF-results. In comparison with the stomacher method published by Fatmi and Schaad (1988), our 48 h soaking procedure proved to give similar or sometimes better results with respect to the number of cfu of Cmm recovered from seed samples. Moreover, the reading of plates was generally easier because fewer saprophytes were observed.

IF and dilution plating used in a single detection method both complement each other, IF is very sensitive and dilution plating is very specific. How to use the results of both tests very much depends on the question whether a prediction of seed health or a prediction of disease in the field is needed. Further, it depends whether bacterial canker is considered to be a quarantine disease, in which case it is highly necessary to be absolutely sure of the result. IF usually gives a higher prediction of 'health' and dilution plating usually gives a higher prediction of 'disease' (Van Vuurde et al., 1991).

The number of seeds to be tested varies from 10 000 (Fatmi and Schaad, 1989) to 30 000 seeds (Gitaitis, 1990) and largely depends on the sensitivity of the method, cultural practices in the field and glasshouse, and the acceptable level of the disease (Geng et al., 1983). When field-grown plants are clipped and moved during thinning and transplanting the risk for spread of the pathogen is large (Chang et al., 1991). To prove absence of the pathogen in 10 000 seeds with high probability, several samples of 10 000 seeds should be tested, as is done for the detection of *Xanthomonas campestris* pv. *campestris* in crucifer seeds in the Netherlands (Franken et al., 1991).

Acknowledgements

Thanks are due to Drs H. Bolkan, J. Janse and R. Bino for critically reading the manuscript and for helpful suggestions. We thank seed companies for providing seed lots. This study was financed by the Department for the Environment, for Quality and Nutrition (MKV) of the Ministry of Agriculture, Nature Management and Fisheries.

References

Bryan, M.K., 1930. Studies on bacterial canker of tomato. Journal of Agricultural Research 41: 825–851.

Chang, R.J., Ries, S.M. and Pataky, J.K., 1991. Dissemination of *Clavibacter michiganensis* ssp. *michiganensis* by practices used to produce tomato transplants. Phytopathology 81: 1276–1281.

Dhanvantari, B.N., 1987. Comparison of selective media for isolation of *Clavibacter michiganense* pv. *michiganense*. Phytopathology 77: 1694 (Abstract).

Dhanvantari, B.N., 1989. Effect of seed extraction methods and seed treatments on control of tomato bacterial canker. Canadian Journal of Plant Pathology 11: 400–408.

Fatmi, M. & Schaad, N.W., 1988. Semiselective agar medium for isolation of *Clavibacter michiganense* ssp. *michiganense* from tomato seed. Phytopathology 78: 121–126.

Fatmi, M. & Schaad, N.W., 1989. Detection of *Clavibacter michiganense* ssp. *michiganense* in tomato seed. In: Saettler, A.W., Schaad, N.W. & Roth, D.A. (Eds), Detection of bacteria in seed and other planting material. The American Phytopathological Society, Minnesota, USA. p. 45–49.

Fatmi, M. & Schaad, N.W. & Bolkan, H.A., 1991. Seed treatments for eradicating *Clavibacter michiganensis* ssp. *michiganensis* from naturally infected tomato seeds. Plant Disease 75: 383–385.

Franken, A.A. & Van der Zouwen, P.S., 1988. Isolation of *Clavibacter michiganensis* ssp. *michiganensis* from tomato seeds by vacuum extraction. In: Abstracts of papers. 5th International Congress of Plant Pathology, Kyoto, Japan, 1988. p. 413.

Franken, A.A.J.M., Van Zeijl, C., Van Bilsen, J.G.P.M., Neuvel, A., De Vogel, R., Van Wingerden, Y., Birnbaum, Y., Van Hateren, J., Van der Zouwen, P.S., 1991. Evaluation of a plating assay for *Xanthomonas campestris* pv. *campestris*. Seed Science and Technology 19: 215–226.

Geng, S., Campbell, R.N., Carter, M., & Hills, F.J., 1983. Quality-control programs for seedborne pathogens. Plant Disease 67: 236–242.

Gitaitis, R.D., 1990. Survey of phytopathogenic bacteria recovered from commercial tomato seed and their secondary spread within tomato transplant fields. Proceedings of the 7th International Conference on Plant Pathogenic Bacteria. Budapest, Hungary, 1989. p. 293–298.

Gitaitis, R.D. & Beaver, R.W. & Dhanvantari, B.N., 1989. Detection of *Clavibacter michiganense* ssp. *michiganense* in tomato transplants. In: Saettler, A.W., Schaad, N.W. & Roth, D.A. (Eds), Detection of bacteria in seed and other planting material. The American Phytopathological Society, Minnesota, USA. p. 116–122.

Gitaitis, R.D. & Beaver, R.W. & Voloudakis, A.E., 1991. Detection of *Clavibacter michiganensis* ssp. *michiganensis* in symptomless tomato transplants. Plant Disease 75: 834–838.

Gross, D.C. & Vidaver A.K., 1979. A selective medium for isolation of *Corynebacterium nebraskense* from soil and plant parts. Phytopathology 69: 82–87.

ISTA, 1988. Report of the 19th International Seminar on Seed Pathology. International Seed Testing Association, Zürich, Switzerland. p. 15.

Maddox, D.A. & Hubbard, J.P., 1990. A method for the detection of seedborne bacterial pathogens in tomato. Phytopathology 80: 1001 (Abstract).

Moffett, M.L., Fahy, P.C. & Cartwright, D., 1983. II. *Corynebacterium michiganense* ssp. *michiganense*. Bacterial canker of tomato and capsicum. In: Fahy, P.C. & Persley, G.J. (Eds), Plant bacterial diseases. p. 46–51.

Roth , D.A., 1989. Review of extraction and isolation methods. In: Saettler, A.W., Schaad, N.W. & Roth, D.A. (Eds), Detection of bacteria in seed and other planting material. The American Phytopathological Society, Minnesota, USA. p. 3–8.

Schaad, N.W., 1982. Detection of seedborne bacterial plant pathogens. Plant Disease 66: 885–890.

Schaad, N.W., 1989. Detection of *Xanthomonas campestris* pv. *campestris* in crucifers. In: Saettler, A.W., Schaad, N.W. & Roth, D.A. (Eds), Detection of bacteria in seed and other planting material. The American Phytopathological Society, Minnesota, USA. p. 73.

Van Vaerenbergh, J.P.C. & Chauveau J.F., 1987. Detection of *Corynebacterium michiganense* in tomato seed lots. Bulletin OEPP/EPPO Bulletin 17: 131–138.

Van Vuurde, J.W.L. & Van den Bovenkamp, G.W., 1987. Working sheet no. 65. *Pseudomonas syringae* pv. *phaseolicola*. ISTA Handbook on Seed Health Testing. International Seed Testing Association, Zürich, Switzerland. 8 pp.

Van Vuurde, J.W.L. & Van den Bovenkamp, G.W. & Birnbaum Y., 1983. Immunofluorescence microscopy and enzyme-linked immunosorbent assay as potential routine tests for the detection of *Pseudomonas syringae phaseolicola* and *Xanthomonas campestris* pv. *phaseoli* in bean seed. Seed Science and Technology 11: 547–559.

Van Vuurde, J.W.L., Franken, A.A.J.M., Birnbaum, Y.E. & Jochems, G., 1991. Characteristics of immunofluorescence microscopy and of dilution-plating to detect *Pseudomonas syringae* pv. *phaseolicola* in bean seed lots and for risk assessment of field incidence of halo blight. Netherlands Journal of Plant Pathology 97: 233–244.

Vruggink, H. & Maas Geesteranus, H.P., 1975. Serological recognition of *Erwinia carotovora* var. *atroseptica*, the causal organism of potato black-leg. Potato Research 18: 546–555.

Waters, C.M. & Bolkan, H.A., 1992. An improved semi selective medium and method of extraction for detecting *Clavibacter michiganensis* ssp. *michiganensis* in tomato seeds. Phytopathology 82: 1072 (Abstract).