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ORIGINAL ARTICLE

Evaluation of the efficacy of immunomagnetic separation for the detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seeds

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bacterial canker, *Clavibacter michiganensis* subsp. *michiganensis*, IMS-plating, seed-borne pathogen, tomato seed extract.

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Abstract

Aims: To evaluate the effectiveness of the optimized immunomagnetic separation (IMS)-plating protocol in relation to other culture, serological and molecular techniques currently used for *Clavibacter michiganensis* subsp. *michiganensis* in seed-testing laboratories.

Methods and results: Bacterial suspensions, tomato seed extracts spiked with the pathogen and naturally infected seeds were IMS-plated for the detection of *C. m.* subsp. *michiganensis*. These results were compared with plating on general (YPGA) and semiselective (mSCM) media, double-antibody sandwich enzyme-linked immunosorbent assay (DAS-ELISA), immunofluorescent assay (IF) or polymerase chain reaction (PCR). Different seed lots and pathogen strains were also tested. IMS-plating allowed the detection of less than 10 CFU ml^{-1} of pathogen in all assayed samples. The mSCM medium provided positive results for 10 CFU ml^{-1} in naturally infected seeds, but up to 14 days was necessary for the typical colonies of the target to be come visible. By serological techniques, 10^3 and up to 10^4 CFU ml^{-1} were detected by IF and ELISA, respectively. DNA extraction was required to obtain positive results by PCR in seed extracts containing 10^3 CFU ml^{-1} or more.

Conclusions: Among the evaluated methods, IMS-plating provided the best results regarding sensitivity and specificity for *C. m.* subsp. *michiganensis* detection, allowing the recovery of viable bacteria from seed extracts.

Significance and impact of the study: IMS-plating increases isolation rates of *C. m.* subsp. *michiganensis* and could improve standard protocols currently used for routine analysis.

Introduction

Clavibacter michiganensis subsp. *michiganensis* (Smith) Davis *et al.* causes vascular wilt, cankers, and leaf and fruit spots on tomato plants (Bradbury 1986). Bacterial canker is considered the most important bacteriosis of tomato and one of its most serious diseases (OEPP/EPPO 2005). It produces severe losses both in greenhouse and field tomato crops with important reductions in yield (Gleason *et al.* 1993). Seeds are the main long-distance passive vector and commerce has facilitated the worldwide distribution of the disease (Thyr *et al.* 1973; Van Vaerenbergh and Chauveau 1987; Gitaitis *et al.* 1992). Protective measures against introduction and spread of this bacterium into the European Community (EC) include it as a quarantine organism (Anonymous 2000) whose presence is prohibited on plants or plant products intended for planting. In spite of the special requirements

established for the introduction and movement of tomato seeds (Anonymous 2000), *C. m.* subsp. *michiganensis* has been introduced into several EC countries. Seed testing is an important control measure but can be extremely difficult as only a few cells of the pathogen, enough to trigger an epidemic, may be present in commercial seed lots (Chang *et al.* 1991; Gitaitis *et al.* 1991). The development of new techniques and validated protocols becomes essential for detection of contaminated seeds, especially in international trade. These techniques must be compared with standard methods before they are widely accepted by diagnosticians (Alvarez 2004). In this way, plating on semiselective media followed by identification of suspected colonies has been the main procedure for detecting *C. m.* subsp. *michiganensis* in tomato seeds, as it must be isolated from plant material to confirm a positive diagnosis (OEPP/EPPO 2005; ISHI Manual of seed health testing methods 2005). However, isolation from seed samples is often difficult because the bacterium grows slowly and more than 10 days may be necessary for its primary isolation (Gleason *et al.* 1993). Additionally, other seed-borne micro-biota may grow faster and *C. m.* subsp. *michiganensis* can be inhibited by overgrowth or competition resulting in false negative diagnoses. Other rapid tests like enzyme-linked immunosorbent assay (ELISA), immunofluorescent assay (IF) or polymerase chain reaction (PCR) are generally suggested for screening or additional confirmation after bacterial isolation, as they do not give information about cell viability.

Immunomagnetic separation followed by plating (IMS-plating) is a combination of immunological and cultural methods that applies serological recognition to trap target cells, which are later cultivated on nonselective media. It has been proposed as a suitable method for testing for *C. m.* subsp. *michiganensis* in tomato seed samples (De León *et al.* 2006). The technique uses spherical magnetic beads directly or indirectly coated with specific antibodies to selectively trap the target bacteria from liquid samples or extracts. These immunomagnetic beads (IMBs) are separated from biotic or abiotic constituents of the sample by utilizing magnetic fields and so the target bacteria can be concentrated.

The purpose of this work was to evaluate the effectiveness of IMS-plating for the detection of *C. m.* subsp. *michiganensis* in tomato seeds compared with other techniques currently used in standard protocols, by using bacterial suspensions and artificially and naturally contaminated seed extracts obtained from pelleted and unpelleted seeds.

Materials and methods

Evaluation of different methods for *C. m.* subsp. *michiganensis* detection in bacterial suspensions and seed extracts

Sensitivity of different detection methods was evaluated following the scheme showed in Fig. 1. The samples analysed were: (i) phosphate buffer saline 0·1 mol l^{-1} pH 7·2 (PBS) spiked with *C. m.* subsp. *michiganensis* (strain IVIA 613, IVIA Plant Pathogenic Bacteria Collection, Spain) to obtain a final concentration ranging from $2{\cdot}2 \times 10^6$ to 2·2 CFU ml^{-1}; (ii) tomato seed extracts experimentally contaminated with the same bacterial strain with a concentration ranging from $1{\cdot}5 \times 10^6$ to 1·5 CFU ml^{-1}; and (iii) seed extracts naturally contaminated with *C. m.* subsp. *michiganensis*, diluted from $7{\cdot}0 \times 10^4$ to 7 CFU ml^{-1}. The assays were run three times.

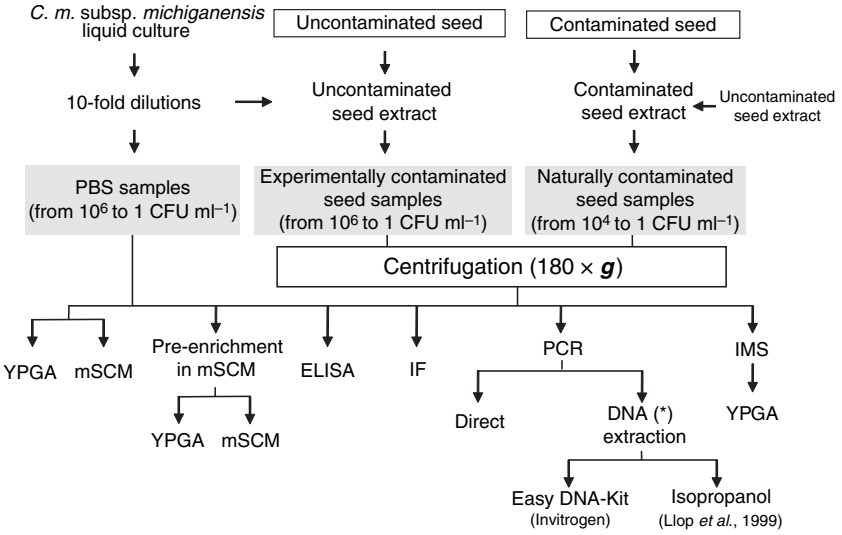

Figure 1 Scheme of experimental procedures to establish the sensitivity of each technique. (*) Only for spiked samples.

Sample preparation

PBS suspensions of *C. m.* subsp. *michiganensis* were prepared with strain IVIA 613 cultivated in 50 ml of yeast-peptone-glucose broth (YPG: yeast extract, 5 g; bactopeptone, 5 g; glucose, 10 g; in 1 litre of distilled water) for 24 h at 25°C on an orbital shaker and diluted in PBS to an optical density of 0·25–0·3 at 550 nm (approximately 10^8 CFU ml^{-1}). Fifty microlitres of the appropriate 10-fold dilution were used to inoculate 5 ml of PBS from $2{\cdot}2 \times 10^6$ to 2·2 CFU ml^{-1}.

Seed extract samples were prepared using previously tested noninfected commercial tomato seeds (cv. Tres Cantos) and naturally infected seeds obtained from plants (cv. Boludo) affected by bacterial canker. All seed extracts were obtained by gently shaking 10 g of tomato seeds in 50 ml of PBS containing 0.1% Tween 20 for 15 min and then soaked at 4°C for 18 h. After removing the supernatant, the seeds were crushed with a pestle in an extraction bag provided with a synthetic intermediate mesh (Bioreba Ag, Reinach, Switzerland) and resuspended in the same supernatant. The largest seed debris was retained in the bag and seed extracts were recovered and plated on YPGA to determine the concentration of seed-borne micro-organisms and, for naturally contaminated seeds, of *C. m.* subsp. *michiganensis*. Uncontaminated seed extracts were spiked with *C. m.* subsp. *michiganensis* as described above. The naturally contaminated seed extract was analysed and the population size of *C. m.* subsp. *michiganensis* was of $7{\cdot}0 \times 10^4$ CFU ml^{-1}. Then, it was 10-fold diluted with the extract from noninfected seeds to achieve a range of pathogen concentration from 7×10^4 to 7 CFU ml^{-1}. Seed extract samples were centrifuged at 180 ***g*** for 5 min to remove debris before applying of IMS, IF, ELISA and PCR techniques.

Standard cultural methods

Forty microlitres of the samples were spread (with a Drigalski spatula) and streaked on YPGA and the semiselective medium mSCM (Waters and Bolkna 1992). Plates of YPGA and mSCM medium were incubated for 4 and 14 days, respectively, at 25°C and typical colonies of *C. m.* subsp. *michiganensis* and nontarget bacterial colonies were counted. To confirm the identity of putative *C. m.* subsp. *michiganensis* randomly selected colonies were tested by nitrocellulose membrane ELISA (De León *et al.* 2006) and by standard identification tests (Dye and Kemp 1977).

Additionally, samples were incubated for 72 h at 25°C in mSCM broth (1 ml sample plus 1 ml of double concentrated mSCM) to selectively enrich them in *C. m.* subsp. *michiganensis*, and plated as described above.

Immunomagnetic separation followed by plating

IMBs precoated with sheep anti-rabbit IgG (Dynabeads M-280; Dynal Biotech, Oslo, Norway) were coated with a specific antiserum for *C. m.* subsp. *michiganensis* (anti-Cmm Pab-PRI; Plant Research International, B.V. Prime Diagnostics, Wageningen, the Netherlands), as previously described (De León *et al.* 2006). Anti-Cmm coated IMBs were added to 1 ml of sample to obtain 10^6 IMBs ml^{-1}. After 1 h of incubation at room temperature with gentle shaking, IMBs were retained using a magnetic particle concentrator (MPC-s, Dynal), washed four times for 10 min each time with 1 ml of PBS containing 0.1% bovine serum albumin (PBS-BSA) and 0.05% Tween 20, and resuspended in 0·1 ml of PBS. After IMS, 40 μl were plated on YPGA, and the target and total bacteria were counted and *C. m.* subsp. *michiganensis* was identified as described above.

Immunofluorescent assay

Samples were diluted 1/10 and 1/100 in sterile distilled water before IF and antisera anti-Cmm Pab-PRI (1/1000 diluted in PBS) and goat anti-rabbit IgG FITC (Sigma-F0382, Sigma-Aldrich, Madrid, Spain; 1/80 diluted in PBS) were used following standard protocols (OEPP/EPPO 2005; ISHI Manual of seed health testing methods 2005). Slides were observed using an epifluorescence microscope (Diaplan, Leitz; Wetzlar, Germany) and cell concentrations calculated as described (Anonymous 1998).

Double-antibody sandwich enzyme-linked immunosorbent assay

A commercial DAS-ELISA kit with polyclonal antibodies recommended by diagnostic protocols of OEPP/EPPO (2005) for *C. m.* subsp. *michiganensis* (Cmm-PathoScreen kit, AGDIA, Inc., Elkhart, IN) was used. The test was performed in antibody-coated 96-well microtitre plates following the manufacturer's indications. Plates were incubated for 2 h at room temperature and read at 405 nm in a BIO-TEK EL×800 multiscan. Absorbance values twice as high as negative controls (PBS or seed extract without *C. m.* subsp. *michiganensis*) were considered positive.

Direct PCR

The PCR procedure was carried out following standard protocols (OEPP/EPPO 2005) using forward primer CMM5 and the reverse primer CMM6, which resulted in a 614-bp product (Dreier *et al.* 1995). Bacterial DNA was amplified in a final volume of 50 μl of a reaction mixture containing 5 μl of 10× PCR buffer, 1.5 mmol l^{-1} $MgCl_2$ (Invitrogen, Carlsbad, CA), 0·2 mmol l^{-1} dNTP mixture (Sigma-Aldrich), 0·5 μmol l^{-1} of each specific primer (Bonsai Technologies Inc., Madrid, Spain), 2 U of *Taq* DNA polymerase (Invitrogen), and 5 μl of sample. PBS and a seed extract nonspiked with *C. m.* subsp. *michiganensis* were used as negative controls. Amplification was performed with a DNA thermal cycler (Perkin Elmer DNA Thermal Cycler 480) with an initial denaturation at

94°C for 3 min; 40 cycles of 94°C for 1 min, 55°C for 1 min, and 72°C for 1 min. After the final reaction cycle, the mixture was held at 72°C for 10 min and stored at 4°C. Aliquots (15 μl) of PCR products were resolved by electrophoresis (100 V) in a 1·2% agarose gel and DNA fragments stained with ethidium bromide (1 μg ml^{-1}) and visualized under UV light.

Further, tomato seed extracts obtained and processed as previously described were diluted 10-fold up to 1/100 000 in PBS to confirm the presence of PCR inhibitors. Undiluted and diluted extracts were spiked with the pathogen (IVIA 613) to obtain 3.4×10^5 CFU ml^{-1}, and direct PCR was performed as previously.

Detection of *C. m.* subsp. *michiganensis* in tomato seed extracts by PCR after DNA extraction

DNA was extracted from PBS suspensions and seed extracts spiked with *C. m.* subsp. *michiganensis* ($3{\cdot}4 \times 10^6$ to 3·4 CFU ml^{-1}) using the Easy-DNA kit from Invitrogen (protocol #3) or the isopropanol extraction method described by Llop *et al.* (1999). PCR amplification results were compared with those obtained by direct PCR. These assays were done in quadruplicate.

PCR was also tested on seed washing extracts spiked with the pathogen to obtain a range of concentration from $2{\cdot}4 \times 10^6$ to 2·4 CFU ml^{-1}. Such extracts were obtained by shaking 10 g of tomato seeds in 50 ml of PBS containing 0.1% Tween 20 for 15 min and soaking at 4°C for 18 h. The extracts were sieved to remove the seeds and then used for PCR: i) directly, ii) after heating to 95°C for 15 min, and iii) after DNA extraction using the isopropanol extraction method (Llop *et al.* 1999). These assays were done in triplicate.

Detection of *C. m.* subsp. *michiganensis* in spiked tomato seed lots

Seed extracts were obtained from ten commercial lots including unpelleted seeds (cv. Boludo, Marglobe, Marmande, Marmande pretreated with thiram, Roma and Tres Cantos) and pelleted seeds (cv. Beaufort, Birloque, Maxifort, and Trinity). Seeds were processed as previously described and spiked with *C. m.* subsp. *michiganensis* (IVIA 613) to obtain a range of concentrations from $1–3 \times 10^3$ to $1–3 \times 10$ CFU ml^{-1}. Pelleted seeds needed pretreatment to remove the coat. Thus, 70 g of seeds were crushed, beaten with a rotary mixer and washed with sterile distilled water and dried at 25°C for 48 h before they were processed. The samples were analysed by plating on mSCM medium, IMS-plating in YPGA and IF as previously described. The assays were done in triplicate.

Detection of different strains of *C. m.* subsp. *michiganensis* in spiked seed extracts

Seed extracts from commercial tomato seeds (cv. Tres Cantos) were spiked with one of five strains of the pathogen: IVIA-2686.1.1 (from Spain), 257 (from Chile), DGPC.PO-103 (from Portugal) PRI 255 (from the Netherlands), and RG 87.2 (from USA). The concentration of bacteria ranged from $1–4 \times 10^3$ to $1–4 \times 10$ CFU ml^{-1}, and seed extracts were analysed as previously indicated. The assays were done in triplicate.

Results

Detection of *C. m.* subsp. *michiganensis* in PBS and spiked seed extracts

Table 1 shows the results using different techniques to analyse bacterial suspensions in PBS. By direct plating on YPGA and mSCM, the bacterium was always detected at concentrations of 10^2 CFU ml^{-1} and over, and even sometimes at 10 CFU ml^{-1}. Its characteristic colonies were observed on YPGA after 4 days of incubation, and it took up to 14 days for their development on mSCM. When a pre-enrichment step in mSCM broth was added before plating on YPGA and mSCM, the pathogen was detected at less than 10 CFU ml^{-1}. IMS followed by plating on YPGA also allowed us to detect less than 10 CFU ml^{-1}. The minimum concentration of bacteria detected by IF was 10^2 CFU ml^{-1} in only one out of three repetitions. The worst sensitivity of the evaluated methods (10^5 CFU ml^{-1}) was obtained with the DAS-ELISA PathoScreen kit. By direct PCR, primers CMM5 and CMM6 provided an approximately 614-pb product from genomic DNA of the bacterium in all samples containing 10^4 CFU ml^{-1} of target cells or more. One out of three repetitions containing 10^3 CFU ml^{-1} (approximately 10 CFU in each PCR reaction tube) gave a faint band on agarose gel after electrophoresis.

Table 1 also shows the sensitivity of different techniques for detection of *C. m.* subsp. *michiganensis* in tomato seed extracts spiked with the pathogen in the same range of concentrations as in PBS. Positive results were obtained when seed extracts were streaked on YPGA in one out of the three repetitions at 10^5 and 10^6 CFU ml^{-1}. At all other concentrations, YPGA showed confluent growth of other seed-borne micro-organisms, and colonies of *C. m.* subsp. *michiganensis* were not observed. When samples were plated on mSCM, positive results were obtained at 10^2 CFU ml^{-1} or more. The medium reduced the number of nontarget micro-organisms by approximately two orders of magnitude (Table 2). However, pre-enrichment of samples in mSCM broth before

Table 1 Detection of *Clavibacter michiganensis* subsp. *michiganensis* in phosphate buffer saline (PBS) suspensions and seed extracts experimentally contaminated with the pathogen from $2{\cdot}2 \times 10^6$ to 2·2 CFU ml^{-1} and from $1{\cdot}5 \times 10^6$ to 1·5 CFU ml^{-1}, respectively. Each experiment was run three times*

Detection techniques†	*C. m.* subsp. *michiganensis* (CFU ml^{-1})													
	10^6	10^5	10^4	10^3	10^2	10	1	10^6	10^5	10^4	10^3	10^2	10	1
	PBS suspensions							Seed extracts‡						
YPGA	+	+	+	+	+	+/−	−	+/−	+/−	−	−	−	−	−
mSCM	+	+	+	+	+	+/−	−	+	+	+/−	+/−	+/−	−	−
YPGA-e	+	+	+	+	+	+	+	−	−	−	−	−	−	−
mSCM-e	+	+	+	+	+	+	+	−	−	−	−	−	−	−
IMS-plating	+	+	+	+	+	+	+	+	+	+	+	+	+	+/−
IF	+	+	+	+	+/−	−	−	+	+	+	+/−	−	−	−
DAS-ELISA	+	+	−	−	−	−	−	+	+	−	−	−	−	−
Direct PCR	+	+	+	+/−	−	−	−	−	−	−	−	−	−	−

*Results are expressed as + (positive detection in the three repetitions), − (negative detection in the three repetitions); +/− (positive detection in one or two out of three repetitions).

†Detection techniques: YPGA, plating on yeast peptone glucose agar; mSCM, plating on semi-selective agar medium; YPGA-e, YPGA after pre-enrichment in mSCM broth; mSCM-e, mSCM after pre-enrichment in mSCM broth; IMS-plating, immunomagnetic separation before plating on YPGA; IF, immunofluorescence; DAS-ELISA, double antibody sandwich ELISA; Direct PCR, direct polymerase chain reaction.

‡Non-target seed associated micro-biota $2{\cdot}9 \times 10^5$ CFU ml^{-1}.

plating on YPGA or mSCM resulted in a considerable increase of some nontarget bacteria that prevented growth and detection of *C. m.* subsp. *michiganensis* at all assayed concentrations. IMS before plating on YPGA reduced the presence of nontarget seed micro-biota in samples to less than 0·1% of the original concentration (Table 2) and allowed detection at less than 10 CFU ml^{-1} in two out of three repetitions. The pathogen was detected by IF in samples containing 10^3 CFU ml^{-1} or more. Dilution of extracts (1/10 or 1/100) improved detection, as undiluted extracts were detached from the slide during required washing. The DAS-ELISA kit was as sensitive as in PBS suspensions, with a detection limit of 10^5 CFU ml^{-1}, whereas direct PCR of extracts did not allow the detection of the pathogen at any of the concentrations assayed.

Detection of *C. m.* subsp. *michiganensis* in naturally infected tomato seeds

Table 3 shows the results of the evaluated methods detecting *C. m.* subsp. *michiganensis* in naturally infected tomato seeds. Colonies of target bacterium appeared on

Table 2 Isolation of *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) on mSCM medium and by immunomagnetic separation before plating on YPGA (IMS-plating) from phosphate buffer saline (PBS) suspensions and experimentally or naturally contaminated tomato seed extracts

Samples	Initial concentration of saprophytes (CFU ml^{-1})	Initial concentration of Cmm (CFU ml^{-1})	Recovered cells*			
			mSCM		IMS-plating	
			Cmm	Saprophytes	Cmm	Saprophytes
PBS suspensions	0	$2{\cdot}2 \pm 0{\cdot}2 \times 10^3$	$2{\cdot}1 \pm 0{\cdot}3 \times 10^3$	0	>10^2†	0
		$2{\cdot}2 \pm 0{\cdot}2 \times 10^2$	$1{\cdot}9 \pm 1{\cdot}3 \times 10^2$	0	$1{\cdot}5 \pm 0{\cdot}2 \times 10^2$	0
		$2{\cdot}2 \pm 0{\cdot}2 \times 10$	$2{\cdot}0 \pm 1{\cdot}4 \times 10$	0	$1{\cdot}3 \pm 0{\cdot}1 \times 10$	0
		$2{\cdot}2 \pm 0{\cdot}2$	0	0	$1{\cdot}3 \pm 0{\cdot}4$	0
Experimentally contaminated extracts	$2{\cdot}9 \pm 1{\cdot}4 \times 10^5$	$1{\cdot}5 \pm 0{\cdot}2 \times 10^3$	$2{\cdot}1 \pm 2{\cdot}5 \times 10^2$	$3{\cdot}8 \pm 2{\cdot}6 \times 10^3$	$5{\cdot}8 \pm 1{\cdot}3 \times 10^2$	$1{\cdot}1 \pm 0{\cdot}6 \times 10^2$
		$1{\cdot}5 \pm 0{\cdot}2 \times 10^2$	$5{\cdot}7 \pm 6{\cdot}9 \times 10$	$4{\cdot}0 \pm 2{\cdot}2 \times 10^3$	$7{\cdot}5 \pm 4{\cdot}2 \times 10$	$2{\cdot}5 \pm 1{\cdot}2 \times 10^2$
		$1{\cdot}5 \pm 0{\cdot}2 \times 10$	0	$3{\cdot}1 \pm 1{\cdot}9 \times 10^3$	$8{\cdot}3 \pm 0{\cdot}1$	$1{\cdot}3 \pm 0{\cdot}9 \times 10^2$
		$1{\cdot}5 \pm 0{\cdot}2$	0	$3{\cdot}9 \pm 2{\cdot}2 \times 10^3$	$2{\cdot}5 \pm 1{\cdot}8$	$1{\cdot}0 \pm 0{\cdot}6 \times 10^2$
Naturally contaminated extracts	$4{\cdot}8 \pm 0{\cdot}8 \times 10^4$	$7{\cdot}0 \pm 1{\cdot}9 \times 10^3$	$5{\cdot}4 \pm 2{\cdot}2 \times 10^3$	$2{\cdot}8 \pm 1{\cdot}1 \times 10^2$	$8{\cdot}4 \pm 4{\cdot}1 \times 10^2$	0
		$7{\cdot}0 \pm 1{\cdot}9 \times 10^2$	$9{\cdot}5 \pm 3{\cdot}8 \times 10^2$	$3{\cdot}4 \pm 1{\cdot}2 \times 10^2$	$2{\cdot}0 \pm 1{\cdot}4 \times 10^2$	0
		$7{\cdot}0 \pm 1{\cdot}9 \times 10$	$6{\cdot}7 \pm 2{\cdot}7 \times 10$	$2{\cdot}7 \pm 0{\cdot}4 \times 10^2$	$1{\cdot}2 \pm 0{\cdot}7 \times 10$	$0{\cdot}8 \pm 1{\cdot}0 \times 10$
		$7{\cdot}0 \pm 1{\cdot}9$	0	$1{\cdot}2 \pm 0{\cdot}8 \times 10^2$	$1{\cdot}0 \pm 0{\cdot}7$	0

*Values presented are means (± SE) for three repetitions.

†Confluent growth of *C. m.* subsp. *michiganensis* on the plates.

Table 3 Detection of *Clavibacter michiganensis* subsp. *michiganensis* in 10-fold dilutions of naturally infected seed extract ($7{\cdot}0 \times 10^4$ CFU ml^{-1} of target) in uncontaminated seed extract (containing $4{\cdot}8 \times 10^4$ CFU ml^{-1} of other micro-organisms). Each experiment was run three times*

Detection techniques†	*C. m.* subsp. *michiganensis* (CFU ml^{-1})				
	10^4	10^3	10^2	10	1
YPGA	+	+/−	−	−	−
mSCM	+	+	+	+	−
YPGA-e	+	+/−	−	−	−
mSCM-e	+	+/−	−	−	−
IMS-plating	+	+	+	+	+/−
IF	+	+/−	−	−	−
DAS-ELISA	+/−	−	−	−	−
Direct PCR	−	−	−	−	−

*Results are expressed as + (positive detection in the three repetitions), − (negative detection in the three repetitions); +/− (positive detection in one or two out of three repetitions).
†Detection techniques: YPGA, plating on yeast peptone glucose agar; mSCM, plating on semi-selective agar medium; YPGA-e, YPGA after pre-enrichment in mSCM broth; mSCM-e, mSCM after pre-enrichment in mSCM broth; IMS-plating, immunomagnetic separation before plating on YPGA; IF, immunofluorescence; DAS-ELISA, double antibody sandwich ELISA; Direct PCR, direct polymerase chain reaction.

YPGA and mSCM when the analysed extracts contained at least 10^3 and 10 CFU ml^{-1}, respectively. A pre-enrichment of the samples in mSCM broth before plating on both media did not improve the detection level on YPGA and led to worse results on mSCM plates. Two out of three repetitions with concentrations of less than 10 CFU ml^{-1} were positive by IMS-plating. The semiselective medium and IMS drastically reduced the nontarget cells in samples (Table 2). The bacterium was not detected by IF and DAS-ELISA below 10^3 and 10^4 CFU ml^{-1}, respectively; whereas direct PCR was unable to detect the pathogen in the assayed conditions at any dilution.

Detection of *C. m.* subsp. *michiganensis* by PCR in tomato seed extracts after DNA extraction

Figure 2 shows that when a tomato seed extract was diluted then spiked with *C. m.* subsp. *michiganensis* at 10^5 CFU ml^{-1}, dilutions over 1/100 gave a specific band for the bacterium by direct PCR, whereas undiluted extract and dilutions up to 1/100 did not. DNA extraction prior to PCR amplification improved detection (Table 4) to a consistent level of 10^5 CFU ml^{-1} using Easy-DNA kit for nucleic acid purification, although up to 10^4 and 10^3 CFU ml^{-1} could be detected in some replicates. The isopropanol extraction method for DNA purification (Llop *et al.* 1999) provided positive results from 10^6 and 10^5 CFU ml^{-1} in some replicates (Fig. 3).

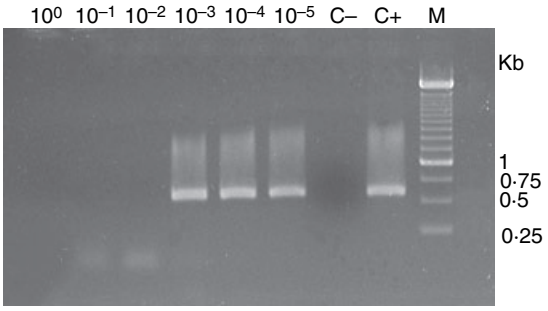

Figure 2 PCR detection of *Clavibacter michiganensis* subsp. *michiganensis* in samples obtained from seed extracts diluted in phosphate buffer saline (from 10^0 to 10^{-5}) and spiked with the pathogen at 10^5 CFU ml^{-1}. Lanes C− and C+ contain negative and positive controls, respectively, and lane M contains the DNA size marker (250 bp ladder; Invitrogen).

Table 4 Detection of *Clavibacter michiganensis* subsp. *michiganensis* by PCR with and without DNA extraction in phosphate buffer saline (PBS) bacterial suspensions and seed extracts spiked with the pathogen (from $3{\cdot}4 \times 10^6$ to 3.4 CFU ml^{-1}). Each experiment was repeated four times*

DNA extraction	*C. m.* subsp. *michiganensis* (CFU ml^{-1})						
	10^6	10^5	10^4	10^3	10^2	10	1
PBS suspensions							
Without extraction	+	+	+	+/−	−	−	−
Isopropanol method†	+	+	+	+/−	−	−	−
Easy-DNA kit‡	+	+	+	+/−	−	−	−
Seed extracts							
Without extraction	−	−	−	−	−	−	−
Isopropanol method	+/−	+/−	−	−	−	−	−
Easy-DNA kit	+	+	+/−	+/−	−	−	−

*Results are expressed as + (positive detection in the four replicates), − (negative detection in the four replicates); +/− (positive detection in some of the four replicates).
†DNA extraction procedure developed by Llop *et al.* (1999).
‡Commercial kit for genomic DNA isolation from Invitrogen Ltd.

When PCR after DNA purification by the isopropanol extraction method was evaluated in seed washing extracts spiked with the pathogen, the detection level was 10^4 CFU ml^{-1}. However, it was not possible to detect *C. m.* subsp. *michiganensis* at any assayed concentration by direct PCR of samples, or after boiling them at 95°C for 15 min (data not shown).

Detection of *C. m.* subsp. *michiganensis* in different tomato seed lots

The results for detection by plating on mSCM, IMS-plating and IF in ten different seed extracts spiked with the pathogen are shown in Table 5. On mSCM medium, the

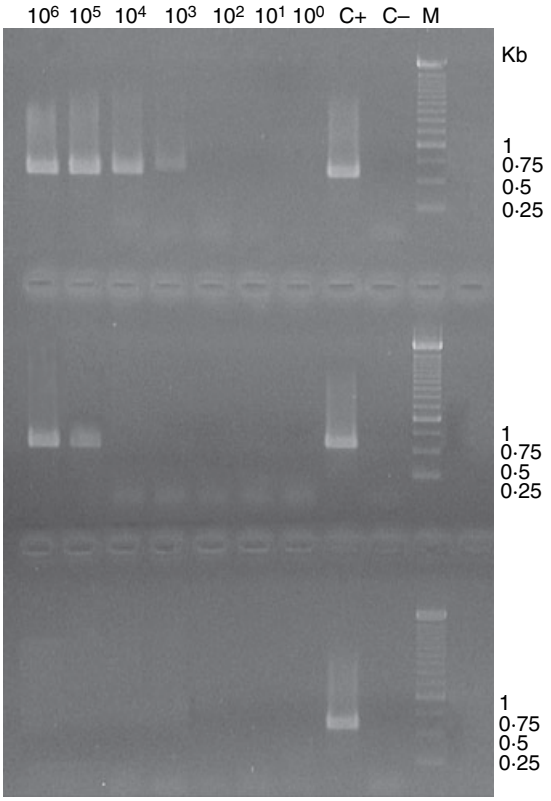

Figure 3 PCR detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seed extract spiked with the bacterium at different concentrations. PCR was performed after DNA extraction with Easy-DNA kit (top), isopropanol extraction method of Llop *et al.* (1999) (middle), and without nucleic acid extraction (below). Lanes C+ and C– contain positive and negative controls, respectively; and lane M contains the DNA size marker (250 bp ladder; Invitrogen).

pathogen was detected in seed extracts from six samples with a sensitivity of 10^2–10 CFU ml^{-1}, but the results were negative in the four extracts obtained from pelleted seeds which had a high population of other associated micro-biota (from $1{\cdot}2 \times 10^5$ to $1{\cdot}7 \times 10^6$ CFU ml^{-1}). IMS before plating on YPGA allowed the detection of the target in all seed extracts at 10 CFU ml^{-1}, although this was achieved in one out of three replicates in two pelleted seed extracts. IF gave a positive reaction at 10^3 CFU ml^{-1} in five extracts (four of them pelleted seeds), but other bacteria different in size and shape to *C. m.* subsp. *michiganensis* also reacted with antiserum in the other five. As described above, at least 10-fold dilution of extracts in distilled water improved the detection by IF.

Detection of different strains of *C. m.* subsp. *michiganensis* in tomato seed extracts

Pathogen was detected in tomato seed extracts spiked with five different strains at a concentration range from 1–4 $\times$ 10^3 to 1–4 $\times$ 10 CFU ml^{-1} (Table 6). All the strains of the pathogen were consistently detected at 10^2 CFU ml^{-1} on mSCM. The method also gave positive results with all samples spiked with strain RG-87.2 at 10 CFU ml^{-1}. IMS followed by plating on YPGA allowed the detection of all the strains at all concentration assayed. Detection by IF was always negative at 10^2 CFU ml^{-1}, and at 10^3 CFU ml^{-1} in some replicates of strains 257, PRI-255 and RG-87.2.

Discussion

Immunomagnetic separation has been adopted as a microbiological diagnostic tool mainly for clinical, food and environmental samples (Olsvik *et al.* 1994; Reynolds *et al.* 1999; Deisingh and Thompson 2004). Nevertheless, immunocapture has so far rarely been applied to the detection of phytopathogenic bacteria. To our knowledge, it has been used for *Erwinia carotovora* subsp. *atroseptica* (Van der Wolf *et al.* 1996), *Pseudomonas syringae* pv. *phaseolicola* (Guven and Mutlu 2000), *Xanthomonas axonopodis* pv. *citri* (Hartung *et al.* 1996), *Xylella fastidiosa* (Pooler *et al.* 1997) and the seed-borne pathogens *Acidovorax avenae* subsp. *citrulli* (Walcott and Gitaitis 2000) and *Pantoea ananatis* (Walcott *et al.* 2002). In previous research we optimized the IMS-plating protocol for *C. m.* subsp. *michiganensis* isolation and described its efficiency to detect and isolate the bacterium at low concentrations in tomato seeds. Less than 10 CFU ml^{-1} could be detected, even in the presence of large numbers of non-target bacteria, showing the high sensitivity and specificity of IMS-plating (De León *et al.* 2006).

IMS followed by plating on nonselective medium (YPGA) provided the highest sensitivity among the evaluated methods, confirming the detection of 10 CFU ml^{-1} or less, and typical pathogen colonies were visible after 4 days of incubation. Results by IMS-plating on YPGA were consistently achieved in naturally and experimentally contaminated seed extracts and were not affected by high concentration of saprophytes in samples, the pelleted seed coating or pathogen strains. In contrast, direct plating of seed extracts on YPGA provided inconsistent results and was unable to detect low concentrations.

Direct plating on mSCM medium allowed 10 and 10^2 CFU ml^{-1} to be detected in naturally and experimentally contaminated seed extracts, respectively. However, *C. m.* subsp. *michiganensis* grows very slowly on this medium and cannot be recognized until 14 days after plating. Moreover, detection was less reliable in samples with a high proportion of other seed-borne micro-organisms, as some of them also grew on mSCM. This probably explains why pre-enrichment of seed extracts in mSCM broth, before plating on semiselective or nonselec-

Table 5 Detection of *Clavibacter michiganensis* subsp. *michiganensis* by direct plating on mSCM medium, immunomagnetic separation before plating on YPGA (IMS-plating) and immunofluorescence (IF) in extracts obtained from 10 commercial seed lots spiked with the pathogen. Each experiment was done in triplicate*

Cultivar	Saprophytes (CFU ml^{-1})	*C. m.* subsp. *michiganensis* (CFU ml^{-1})								
		10^3	10^2	10	10^3	10^2	10	10^3	10^2	10
		mSCM			IMS-plating			IF		
Boludo	$3{\cdot}6 \times 10^2$	+	+	–	+	+	+	+/–	–	–
Marglobe	$5{\cdot}3 \times 10^4$	+	+/–	–	+	+	+	cr†	cr	cr
Marmande	$5{\cdot}8 \times 10^3$	+	+/–	+/–	+	+	+	cr	cr	cr
Marmande‡	$4{\cdot}0 \times 10^4$	+/–	+/–	–	+	+	+	cr	cr	cr
Roma	$3{\cdot}7 \times 10^4$	+	+	+/–	+	+	+	cr	cr	cr
Tres Cantos	$5{\cdot}7 \times 10^3$	+	+	+/–	+	+	+	cr	cr	cr
Beaufort§	$1{\cdot}1 \times 10^6$	–	–	–	+	+	+/–	+/–	–	–
Birloque§	$1{\cdot}2 \times 10^5$	–	–	–	+	+	+	+/–	–	–
Maxifort§	$7{\cdot}7 \times 10^5$	–	–	–	+	+	+/–	+/–	–	–
Trinity§	$1{\cdot}7 \times 10^6$	–	–	–	+	+	+	+/–	–	–

*Results are expressed as + (positive detection in the three repetitions), – (negative detection in the three repetitions); +/– (positive detection in one or two of three repetitions).
†Cross reaction.
‡Seed lot treated with thiram.
§Pelleted seeds.

Table 6 Detection of different strains of *Clavibacter michiganensis* subsp. *michiganensis* by direct plating on mSCM medium, immunomagnetic separations before plating on YPGA (IMS-plating) and immunofluorescence (IF) in seed extracts spiked with the pathogen. Each experiment was done in triplicate*

Strains† (Origin)	Saprophytes (CFU ml^{-1})	*C. m.* subsp. *michiganensis* (CFU ml^{-1})	Detection method		
			mSCM	IMS-plating	IF
IVIA-2686.1.1 (Spain)	$6{\cdot}4 \times 10^4$	$1{\cdot}1 \times 10^3$	+	+	+
		$1{\cdot}1 \times 10^2$	+	+	–
		$1{\cdot}1 \times 10$	+/–	+	–
257 (Chile)	$1{\cdot}0 \times 10^4$	$1{\cdot}4 \times 10^3$	+	+	+/–
		$1{\cdot}4 \times 10^2$	+	+	–
		$1{\cdot}4 \times 10$	+/–	+	–
DGPCPO-103 (Portugal)	$6{\cdot}4 \times 10^4$	$1{\cdot}7 \times 10^3$	+	+	+
		$1{\cdot}7 \times 10^2$	+	+	–
		$1{\cdot}7 \times 10$	+/–	+	–
PRI-255 (the Netherlands)	$1{\cdot}0 \times 10^4$	$1{\cdot}8 \times 10^3$	+	+	+/–
		$1{\cdot}8 \times 10^2$	+	+	–
		$1{\cdot}8 \times 10$	+/–	+	–
RG-87.2 (USA)	$6{\cdot}4 \times 10^3$	$3{\cdot}5 \times 10^3$	+	+	+/–
		$3{\cdot}5 \times 10^2$	+	+	–
		$3{\cdot}5 \times 10$	+	+	–

*Results are expressed as + (positive detection in the three repetitions), – (negative detection in the three repetitions); +/– (positive detection in one or two of three repetitions).
†Strains: IVIA-2686.1.1 from the Instituto Valenciano de Investigaciones Agrarias (IVIA) collection; 257, from X. Besoaín; DGPC.PO-103, from M. Eloy; PRI-255, from J. Van der Wolf, Plant Research International; and RG-87.2, from R. D. Gitaitis.

tive media, did not improve the results of direct plating. For the same reason, isolation of *C. m.* subsp. *michiganensis* from pelleted seeds on semiselective medium was not possible at 10^3 CFU ml^{-1} or less because of the high proportion of other micro-organisms, possibly caused by the coating. According to the EPPO protocol (OEPP/EPPO, 2005), the current methods used for detection of this bacterium have not been evaluated in pelleted seeds and should not be used for them. However, this system has been commercially implemented in recent years and the development of techniques for the laboratory analysis of pelleted seeds is required.

The SCM medium (Fatmi and Schaad 1988), and/or its modification (Waters and Bolkna 1992) is recommended and widely used for the isolation of *C. m.* subsp. *michiganensis* (OEPP/EPPO 2005; ISHI Manual of seed health testing methods 2005). However, it has been questioned because its sensitivity was not as described by Fatmi and Schaad (1988), probably due to variations in seed contamination and extraction procedures (Hadas *et al.* 2005). In addition, large variations in the recovery rates of different strains have been reported (Hadas *et al.* 2005), although this was not observed in our work.

Serological techniques such as IF, using polyclonal antibodies (OEPP/EPPO 2005), provided positive results for 10^3 CFU ml^{-1} or more in seed extracts, but sometimes the specificity was low, with cross-reactions in some seed lots. Furthermore, the presence of seed debris masked the appearance of bacteria, as reported for *Ralstonia solanacearum* in potato tuber extracts (Elphinstone *et al.* 1996). In our case, seed extracts diluted 1/10 or 1/100 gave better results than undiluted extracts. Sample preparation by soaking seeds has been also proposed to avoid the release of interfering saprophytes and seed debris (Franken *et al.* 1993). However, the extraction of *C. m.* subsp. *michiganensis* from seeds by blending has been reported as more effective than by soaking (Fatmi and Schaad 1988). Lower sensitivity (10^4 CFU ml^{-1} for naturally infected and 10^5 CFU ml^{-1} for spiked seed extracts) was recorded with a commercial DAS-ELISA test kit although it could be suitable for use in confirmatory tests after pathogen isolation (Alvarez 2004).

Direct PCR detected *C. m.* subsp. *michiganensis* at concentrations of 10^3 CFU ml^{-1} or more in PBS suspensions (approximately 10 cells per PCR reaction mix), but no amplifications were recorded in seed samples. Plant-derived compounds that inhibit the PCR reaction have been well documented in several works (Prosen *et al.* 1993; Dreier *et al.* 1995; Elphinstone *et al.* 1996; Schaad *et al.* 1999; Walcott and Gitaitis 2000; Weller *et al.* 2000; Meng *et al.* 2004), and can lead to a false negative result (Louws *et al.* 1999). Thus, tomato seed samples should be treated to remove or reduce inhibitors, e.g. by dilution or heating of samples (Sousa-Santos *et al.* 1997; Wang *et al.* 1999; Meng *et al.* 2004), selective separation of target cells from inhibitors by IMS (Walcott and Gitaitis 2000; Walcott *et al.* 2002), or DNA extraction (Dreier *et al.* 1995). Pre-enrichment in solid selective media or BIO-PCR may also be successful because inhibitors are diluted or adsorbed and target pathogen levels can increase (Schaad *et al.* 1995).

Dilutions up to 1/1000 of tomato seed extract were necessary to obtain a positive result by direct PCR. This fact reveals the presence of PCR inhibitors in seed extracts as reported by Sousa-Santos *et al.* (1997). In addition, Dreier *et al.* (1995) could only detect *C. m.* subsp. *michiganensis* by PCR after DNA extraction with phenol-chloroform. Surprisingly, Hadas *et al.* (2005) achieved consistent positive results by direct PCR in seed extracts obtained from 2000 noninfected seeds containing a single contaminated tomato seed (<1 cell per PCR reaction mix).

Our results confirmed that DNA extraction from commercial tomato seeds spiked with the pathogen can reduce PCR inhibitors. Both Easy-DNA kit (Invitrogen) and isopropanol extraction allowed PCR amplification of the target fragment, improving detection compared with direct PCR. However, Easy-DNA kit provided higher sensitivity, detecting 10^3 CFU ml^{-1} whereas 10^5 CFU ml^{-1} were detected using isopropanol extraction. It has been reported that more laborious extraction methods provide higher yields of pure DNA, particularly from complex samples (Wilson 1997). The Easy-DNA kit gave a similar detection level to that obtained by Dreier *et al.* (1995) in tomato seed extracts, but it was simpler to use and avoids handling dangerous compounds. Detection by PCR in seed washing extracts was improved by isopropanol extraction when compared to direct PCR. This was proposed as a simple rapid method for extracting DNA from different phytopathogenic bacteria, detecting 10^2–10^3 CFU ml^{-1} in other models (Llop *et al.* 1999), but probably needs some optimization for routine use with blended extracts.

In general, no one method is foolproof in the analysis of seed samples and the use of integrated diagnosis can give more reliable results (Alvarez 2004). Indeed, IMS-plating combines the advantages of serological recognition, sample concentration and growth on culture media, allowing pathogen isolation for further analysis. This comparative study highlights the usefulness of IMS-plating for the isolation and detection of *C. m.* subsp. *michiganensis* in unpelleted or pelleted tomato seeds. It is highly sensitive, easy to perform, confirms the presence of viable bacteria much faster than using semiselective medium, and can be combined with other detection techniques to improve the accuracy of the diagnosis. Validation of IMS-plating in routine analysis and ring-tests should be performed so as to include it as a standard protocol for detecting this quarantine bacterium in tomato seeds. The data presented here suggest its advantages over other techniques.

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