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Detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seeds using immunomagnetic separation

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Abstract

The use of pathogen-free plant material is the main strategy for controlling bacterial canker of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis*. However, detection and isolation of this pathogen from seeds before field or greenhouse cultivation is difficult when the bacterium is at low concentration and associated microbiota are present. Immunomagnetic separation (IMS), based on the use of immunomagnetic beads (IMBs) coated with specific antibodies, was used to capture *C. michiganensis* subsp. *michiganensis* cells, allowing removal of non-target bacteria from samples before plating on non-selective medium. Different concentrations of IMBs and of two antisera were tested, showing that IMS with 10^6 IMBs/ml coated with a polyclonal antiserum at 1/3200 dilution recovered more than 50% of target cells from initial inocula of 10^3 to 10^0 CFU/ml. Threshold detection was lower than 10 CFU/ml even in seed extracts containing seed debris and high populations of non-target bacteria. The IMS permitted *C. michiganensis* subsp. *michiganensis* isolation from naturally infected seeds with higher sensitivity and faster than direct isolation on the semiselective medium currently used and could become a simple viable system for routinely testing tomato seed lots in phytosanitary diagnostic laboratories.

Keywords: *Clavibacter michiganensis* subsp. *michiganensis*; Detection; Bacterial canker; Tomato seeds; Immunomagnetic separation; IMS

1. Introduction

Bacterial canker, caused by *Clavibacter michiganensis* subsp. *michiganensis* (Smith) Davis et al. (*C. michiganensis* subsp. *michiganensis*), is a harmful bacteriosis on tomato crops, causing great economic losses in commercial tomato production areas (Gleason et al., 1993). It is particularly important for tomato seed producers, since infected seeds are considered the primary inoculum source (Thyr et al., 1973; Gitaitis et al., 1992) and current legislation forbids their commercialization. Seed dissemination explains many new outbreaks of bacterial canker and its worldwide distribution. The sporadic but devastating nature of this disease, the capability of *C. michiganensis* subsp. *michiganensis* to survive several months on plant debris

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and seeds under natural conditions (Gleason et al., 1991; Fatmi and Schaad, 2002), and the absence of effective control measures on affected crops, makes it a potential threat for all tomato producing areas.

Therefore, the most important strategy for controlling bacterial canker has been the use of pathogen-free seeds (Rademaker and Janse, 1994; Janse and Wenneker, 2002). In the European Union, tomato seeds must be obtained by an appropriate acid extraction or equivalent method and (a) originate in areas where bacterial canker is not known to occur; or (b) be free from these symptoms on the plants at the place of production during their complete growth cycle; or (c) be certified free from this harmful organism after official testing on a representative sample using appropriate methods. (Anonymous, 2004, Council Directive 2000/29/EC). Despite these measures, new outbreaks have been reported in recent years in several European countries.

Much research has been done to develop sensitive and specific technologies to improve seed-borne pathogen detection and ensure successful control measures. The International Seed Health Initiative for Vegetables (ISHI) and the European and Mediterranean Plant Protection Organization (EPPO) (EPPO/CABI, 2005; ISHI Manual of seed health testing methods, 2005) have proposed plating seed extracts on semiselective media, followed by identification of suspected colonies, as the main procedure for detecting *C. michiganensis* subsp. *michiganensis* in tomato seeds. However, this slow-growing bacterium can be inhibited on culture media by other microorganisms, even using semiselective media, on which its characteristic colonies develop even more slowly, sometimes taking more than 10 days (Gleason et al., 1993).

Other detection techniques currently used for *C. michiganensis* subsp. *michiganensis* are: enzyme-linked immunosorbent assay (ELISA), immunofluorescent assay (IFA), polymerase chain reaction (PCR) or bioassay (inoculating host plants with potentially contaminated extracts) (Franken et al., 1993; Gleason et al., 1993; Dreier et al., 1995). Each of these techniques has advantages and also disadvantages related to their lack of sensitivity and/or specificity, cost or are time consuming, and require chambers under controlled conditions for bioassays. Furthermore, bacterial cells of the target must be isolated and their pathogenicity demonstrated for a positive diagnosis. In fact, rapid and sensitive screening tests for presumptive diagnosis such as IFA or PCR neither give information about viability nor allow the isolation of bacteria.

Immunomagnetic separation (IMS), a variant of the immunoisolation technique, is a process by which immunomagnetic beads (IMBs) are coated with specific antibodies allowing selective trapping of target cells from the sample, afterwards they can be resuspended in a smaller volume of solution and plated on non-selective medium. This method therefore combines the advantages of serologic recognition, target bacteria concentration and growth of characteristic colonies on culture medium, facilitating the isolation required for positive diagnosis. This technique has been tested as a diagnostic tool in clinical microbiology (Nilsson et al., 1996; Stark et al., 1996; Roberts and Hirst, 1997), food microbiology (Blake and Weimer, 1997; Tomoyasu, 1998; Gray and Bhunia, 2005), environmental microbiology (Rochelle et al., 1999; Straub et al., 2005) and veterinary medicine (Biswas et al., 1994; Gagné et al., 1998). Nevertheless, the use of IMS for phytopathogenic bacteria is rarer and, as far as we know, has only 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). It has been reported that utilization of IMS prior to plating on agar media improves sensitivity in detecting and isolating plant pathogenic bacteria from plant material (van der Wolf et al., 1996; Walcott et al., 2002). The IMS procedure has also been combined with PCR, increasing plant pathogen detection sensitivity over direct PCR (Hartung et al., 1996; van der Wolf et al., 1996; Walcott and Gitaitis, 2000). Additionally, comparative studies showed that IMS-plating was more sensitive than IMS-PCR (van der Wolf et al., 1996; Walcott et al., 2002).

Successful recovery of target cells by IMS depends on many factors: antibody specificity, antibody and IMB concentrations, bacterial losses by washing, ionic strength and use of dispersing agents that avoid bead and cell agglomerations (van der Wolf et al., 1996; Grant et al., 1998; Gottschalk et al., 1999; Davies et al., 2003). The aim of this study was to develop and standardize an immunomagnetic separation technique for selective isolation of *C. michiganensis* subsp. *michiganensis* from tomato seeds to improve the specificity and sensitivity with respect to standard isolation of this bacterium.

2. Materials and methods

2.1. Bacterial strains and culture media

C. michiganensis subsp. *michiganensis* strain 613, from the IVIA Collection of Plant Pathogenic Bacteria

(Instituto Valenciano de Investigaciones Agrarias, Spain), was used to optimize and evaluate the IMS technique; it was routinely cultured in yeast–peptone–glucose agar (YPGA: yeast extract, 5 g; bactopeptone, 5 g; glucose, 10 g; agar, 15 g; in 1 l of distilled water). A fluorescent *Pseudomonas* sp. isolated from healthy tomato plants was used for adding to spiked samples. It was cultivated on Pseudomonas Agar F medium (BioLife). The semiselective medium mSCM (Walters and Bolkna, 1992), modified from the SCM (Fatmi and Schaad, 1988) by substituting mannose for sucrose, was used to isolate *C. michiganensis* subsp. *michiganensis* from naturally infected seeds. The plates were incubated at 25 °C.

2.2. Antisera

Anti-Cmm polyclonal antisera were obtained from Plant Research International, B.V. Prime Diagnostics, The Netherlands (anti-Cmm Pab-PRI) and Bio-Rad, Phyto-Diagnostics, France (anti-Cmm Pab-BR).

2.3. Coating of magnetic beads

Immunomagnetic beads (IMBs) pre-coated with sheep anti-rabbit IgG (Dynabeads M-280; Dynal, Norway) were coated with specific antisera for *C. michiganensis* subsp. *michiganensis*. Firstly, the beads were washed three times with phosphate buffer saline 0.1 M pH 7.2 (PBS), collected using a magnetic particle concentrator (MPC-s, Dynal) and resuspended in PBS to obtain the desired concentration. After this, IMBs were incubated with the anti-Cmm antiserum for 24 h at 4 °C with gentle shaking as instructed by the manufacturers, then washed three times with PBS containing 0.1% bovine serum albumin (PBS-BSA) and 0.05% Tween 20 to remove non-attached antibodies. Finally, the beads were resuspended in PBS-BSA to obtain a stock suspension (10^8 IMBs/ml) and stored at 4 °C.

2.4. Standardization of IMS technique

To select the most appropriate antiserum for IMS, the anti-Cmm Pab-PRI and Pab-BR were evaluated using different dilutions (1/200, 1/400, 1/800, 1/1600, 1/3200 and 1/6400) to coat 10^5 IMBs/ml. Then, aliquots of anti-Cmm-coated IMBs were added to 1 ml of *C. michiganensis* subsp. *michiganensis* suspension (5.7×10^3 CFU/ml) to obtain 10^5 IMBs/ml. After 1 h of incubation at room temperature with gentle shaking, the IMBs were washed three times with 1 ml of PBS-BSA containing 0.05% Tween 20 for 10 min. On the final rinse, they were resuspended in 1 ml of PBS and 0.1 ml was spread on YPGA plates. After incubation at 25 °C for 5 days, the *C. michiganensis* subsp. *michiganensis*-like colonies were enumerated to obtain information about recovery rates. Since more than one cell may become attached to a single bead, leading to underestimation of the recovered cells, 0.1 ml of the washing solutions were also plated on YPGA and *C. michiganensis* subsp. *michiganensis* colonies counted to calculate the number of cells lost by washing. The number of cells captured by the beads was estimated by subtracting the number of cells lost by washing from the cells present in the sample before the IMS. Furthermore, a relation between these two estimates was established using the formula: $AI = (No - L)/Cc$, where AI is the aggregation index, No is the initial inoculum, L is the number of cells lost through washing and Cc the recovered cells estimated by colony counting when IMBs were plated on agar plates after the IMS. Aggregation index values of 1 indicate that each colony grown on agar plates was developed from one cell attached to beads.

To establish the optimal concentration of beads and antiserum dilution for IMS, a factorial experiment was carried out. Bead concentrations of 10^4, 10^5, 10^6 and 10^7 IMBs/ml and anti-Cmm Pab-PRI dilutions of 1/800, 1/3200 and 1/6400 were tested following the IMS procedure previously described. In addition, uncoated IMBs were also tested to evaluate the dragging of bacteria non-specifically bound to the beads during the IMS. The assay was conducted using *C. michiganensis* subsp. *michiganensis* suspensions with 6.7×10^3 CFU/ml. The experiment was performed in triplicate. Data of recovered CFU were log-transformed ($\log_{10}(X+1)$) and homogeneity of variance was verified by Bartlett's test prior to analysis (Little and Hills, 1975). Two-way analysis of variance (ANOVA) was performed with transformed data using the Systat Statistical Software Package version 10 (SPSS Inc.). Separation of means was based on Fisher's least significant difference (LSD) test at $p < 0.05$.

2.5. Threshold of cell recovery and specificity of IMS

To determine the lowest population of *C. michiganensis* subsp. *michiganensis* detected by the IMS method, 10-fold dilutions of the bacteria ranging from 2.7×10^3 to 2.7 CFU/ml were prepared in PBS and subjected to IMS. The assay was done in triplicate using 10^6 IMBs/ml and an anti-Cmm Pab-PRI dilution of 1/3200 to ensure optimal IMS conditions (see results). The IMBs were resuspended in 1 ml of PBS after the IMS, and recovery rates estimated by plating 0.1 ml on YPGA

and colony counting. When less than 10^2 CFU/ml was used as initial inoculum, the volume of PBS for resuspending the IMBs was 0.1 ml and the total volume of IMB suspension was plated on agar medium.

To verify the specificity of the method, a bacterial suspension containing a mixture of *C. michiganensis* subsp. *michiganensis* and *Pseudomonas* sp. isolated from tomato used as contaminant bacteria was subjected to IMS. Ten-fold dilutions of *Pseudomonas* sp. (from 2.2×10^6 to 2.2×10^3 CFU/ml) were mixed with *C. michiganensis* subsp. *michiganensis* at a final concentration of $\sim10^2$ CFU/ml. After the IMS, IMBs were plated on Pseudomonas Agar F to be able to distinguish the *Pseudomonas* sp. colonies clearly from those of *C. michiganensis* subsp. *michiganensis* and count them separately for recovery rate calculations. The assay was done in triplicate.

2.6. Effect of tomato seed debris and saprophytic microbiota on cell recovery

To determine the effect of seed debris on the IMB-cell complex formation, IMS was conducted on a tomato seed extract containing seed debris and seed-borne saprophytic microbiota, to which different concentrations of *C. michiganensis* subsp. *michiganensis* were added. Seed extract was generated by gently shaking 5 g of tomato seeds in 50 ml of PBS containing 0.1% Tween 20 on a rotatory shaker for 15 min and then soaked at 4 °C for 18 h. Supernatant was removed and kept and the seeds were deposited in an extraction bag provided with a synthetic intermediate mesh (Bioreba), crushed with a pestle and resuspended in the same supernatant. The largest seed debris fractions were retained in the bag and seed extract was inoculated with *C. michiganensis* subsp. *michiganensis* to obtain 5.1, 5.1×10 and 8.3×10^2 CFU/ml. 100 µl of seed extract were plated on YPGA before and after IMS. After incubation, plates were surveyed for the development of mucoid yellow colonies typical of *C. michiganensis* subsp. *michiganensis* that were counted, besides those of saprophytic bacteria.

To confirm the identity of putative *C. michiganensis* subsp. *michiganensis* colonies, randomly selected colonies were tested by nitrocellulose membrane ELISA (NCM-ELISA). Samples (5µl) of bacterial suspensions of each colony ($\sim10^7$ CFU/ml) were deposited on a nitrocellulose membrane that was dried and blocked with PBS-BSA plus 0.05% Tween 20 for 15 min. Excess liquid was removed and the membrane incubated with anti-Cmm Pab-PRI (1:2500 dilution) for 30 min, washed three times with PBS-BSA containing 0.05% Tween 20 and incubated for 30 min with goat–antirabbit conjugated sera with alkaline phosphatase (Sigma) (1:10,000 dilution). The membrane was washed three times as previously described and then incubated with Sigma Fast 5-bromo-4-chloro-3-indolyl phosphate/nitro blue tetrazolium tablets until a purple colour change occurred in the positive control. All incubations were done with gentle shaking at room temperature.

2.7. Isolation of C. michiganensis subsp. michiganensis from naturally infected seeds by IMS

Detection of *C. michiganensis* subsp. *michiganensis* by IMS was also evaluated on tomato seeds obtained manually from plants affected by bacterial canker. Two grams of seeds were soaked in 20 ml of PBS plus 0.1% Tween 20 and processed following the previously described procedure. The seed extract was diluted 1/100 and 1/10,000 in another extract obtained from seed of non-infected tomato plants and 10 µl of each were directly streaked on YPGA and mSCM. The original extract and both dilutions were subjected to IMS, the recovered IMBs were resuspended in 50 µl of PBS and 10 µl aliquots were streaked on YPGA. The assay was performed in quadruplicate and the identity of suspected

Table 1
Effect of concentration of IMBs and anti-Cmm Pab-PRI on cell recovery and aggregation index, from a 6.7×10^3 CFU/ml *C. michiganensis* subsp. *michiganensis* suspension

Antiserum dilution	Bead concentration	*C. michiganensis* subsp. *michiganensis* recovered cells (CFU/ml) [a, b]	Aggregation index
1:800	10^7	$(6.27\pm0.53)\times10^3$ a	1.2
	10^6	$(5.22\pm0.57)\times10^3$ ab	1.3
	10^5	$(2.07\pm0.83)\times10^3$ bcd	2.5
	10^4	$(1.26\pm0.23)\times10^3$ de	4.2
1:3200	10^7	$(6.14\pm0.44)\times10^3$ a	1.2
	10^6	$(5.33\pm0.46)\times10^3$ ab	1.1
	10^5	$(3.06\pm1.45)\times10^3$ abcd	3.4
	10^4	$(1.87\pm0.75)\times10^3$ cd	9.2
1:6400	10^7	$(4.54\pm0.59)\times10^3$ abc	1.1
	10^6	$(5.23\pm0.97)\times10^3$ ab	1.5
	10^5	$(1.43\pm0.92)\times10^3$ de	3.7
	10^4	$(5.33\pm2.90)\times10^2$ e	4.2
No serum	10^7	(0.33 ± 0.41) g	nd
	10^6	(2.00 ± 0.71) fg	nd
	10^5	(3.00 ± 1.41) fg	nd
	10^4	(4.00 ± 1.87) f	nd

An aggregation index equal to one indicates that each colony is developed from one immunocaptured cell. Data are the average of three replicates.

[a] Mean ± standard error.

[b] Values followed by different letters are different at $p<0.05$ according to LSD test. Data were log-transformed prior to analysis.

Table 2
Recovery rates by immunocapture from bacterial suspension of *C. michiganensis* subsp. *michiganensis* at different concentrations

Initial inoculum size (CFU/ml)	Positives for three samples	Recovered cells after IMS [a] (CFU/ml)
2.7×10^3	3/3	$(1.56\pm0.26)\times10^3$
2.7×10^2	3/3	$(2.26\pm0.17)\times10^2$
2.7×10	3/3	$(1.40\pm0.20)\times10$
2.7	2/3	1.60 ± 0.88

IMS was carried out using 10^6 IMBs/ml coated with anti-Cmm Pab-PRI (1/3200 dilution).
[a] Recovered cells are expressed as the average of three replicates ± standard error.

C. michiganensis subsp. *michiganensis* colonies was confirmed by NCM-ELISA and by standard tests (Dye and Kemp, 1977).

3. Results

3.1. Standardization of IMS technique

When anti-Cmm Pab-PRI was used to coat the IMBs, more than 1.2×10^3 CFU/ml of *C. michiganensis* subsp. *michiganensis* were recovered from a bacterial suspension of 5.7×10^3 CFU/ml, against less than 5×10^2 CFU/ml with anti-Cmm Pab-BR. This reduction in captured cells obtained with anti-Cmm Pab-BR was observed for all sera dilutions assayed.

Table 1 shows the results obtained with different concentrations of both IMBs and anti-Cmm Pab-PRI. Target-cell recovery was significantly affected ($p<0.05$) by the IMBs concentration and the antiserum dilution. Moreover, a significant interaction between the two factors was also observed. The best recovery percentage (93.6% of cells captured from a suspension of 6.7×10^3 CFU/ml) was with 10^7 IMBs/ml coated with anti-Cmm Pab-PRI at a dilution of 1/800. However, this value was not statistically different from that obtained with 10^6 IMBs/ml coated with all antiserum dilutions tested (77.9%, 79.5% and 78.1% for 1/800, 1/3200 and 1/6400 dilutions, respectively). The use of 10^4 IMBs/ml concentration presented a significant decrease in cell recovery in all cases as well as 10^5 IMBs/ml at 1/6400 antiserum dilution. In addition, less than 0.1% of cells were recovered using IMBs at any evaluated concentration without the specific antibodies.

Moreover, it is important to emphasize that at 10^5 and 10^4 IMBs/ml the aggregation index was over 2.5, whereas it was only 1.1 for 10^7 and 10^6 IMBs/ml coated with 1/6400 and 1/3200 antiserum dilutions, respectively (Table 1). In successive assays, IMS was performed with an optimum concentration of 10^6 IMBs/ml and an anti-Cmm Pab-PRI dilution of 1/3200 to cover the spheres.

Table 4
Isolation of *C. michiganensis* subsp. *michiganensis* on YPGA after IMS from tomato seed extracts containing natural saprophytic microbiota and inoculated with different concentrations of target bacteria

Initial inoculum size of *C. michiganensis* subsp. *michiganensis* (CFU/ml)	Recovered cells after IMS [a] (CFU/ml)	
	C. michiganensis subsp. *michiganensis*	Contaminants
8.30×10^2	$(8.10\pm0.43)\times10^2$	$(5.20\pm1.58)\times10$
5.10×10	$(3.46\pm0.23)\times10$	6.60 ± 1.70
5.10	4.33 ± 2.33	$(2.60\pm1.69)\times10$

[a] Recovered cells are expressed as the average of three replicates ± standard error.

3.2. Threshold of cell recovery and specificity of IMS

When the IMS sensitivity was evaluated on samples containing different pathogen concentrations, the number of cells recovered was of the same order of magnitude as the initial inoculum (Table 2) and more than 50% in all cases. It should be noted that the bacterium was detected in two of three samples when the target concentration was as low as 2.7 CFU/ml. In this case, the IMB-bound cells, from 1 ml of sample, were concentrated by resuspending them in 0.1 ml of PBS prior to plating on YPGA.

In the presence of a *Pseudomonas* sp. strain, used as contaminant, more than 89% of *C. michiganensis*

Table 3
Immunomagnetic isolation of *C. michiganensis* subsp. *michiganensis* on YPGA from a suspension containing target cells and a fluorescent *Pseudomonas* sp. strain used as a contaminant at different concentrations

Initial inoculum size (CFU/ml)		Recovered cells after IMS [a] (CFU/ml)	
C. michiganensis subsp. *michiganensis*	Contaminants	*C. michiganensis* subsp. *michiganensis*	Contaminants
6.30×10^2	2.19×10^3	$(5.91\pm0.07)\times10^2$	0
4.30×10^2	2.80×10^4	$(4.13\pm0.30)\times10^2$	$(4.60\pm2.05)\times10$
6.30×10^2	2.19×10^5	$(5.66\pm0.32)\times10^2$	$(6.17\pm8.70)\times10$
6.30×10^2	2.19×10^6	$(5.70\pm0.05)\times10^2$	$(1.07\pm1.37)\times10^2$

[a] Recovered cells are expressed as the average of three replicates ± standard error.

subsp. *michiganensis* cells from 1 ml samples containing 6.3×10^2 CFU/ml were recovered by IMS, even when the *Pseudomonas* sp. population was as high as 2.2×10^6 CFU/ml (Table 3). Indeed, the percentage of *Pseudomonas* sp. recovered never exceeded 0.2% of its initial inoculum. When the non-target cells were at a low concentration (2.2×10^3 CFU/ml), none of them appeared in the plates and only *C. michiganensis* subsp. *michiganensis* colonies grew on agar medium.

3.3. Effect of tomato seed debris and saprophytic microbiota on cell recovery

When *C. michiganensis* subsp. *michiganensis* cells were added to a seed extract containing a natural seed-borne bacterial population, the target pathogen recovery percentages reached 97.6% with an inoculum size of 8.3×10^2 CFU/ml (Table 4). A reduction in the high levels of non-target cells was observed on agar plates after the IMS compared with untreated samples (Fig. 1). When the seed extract was inoculated with a low number of *C. michiganensis* subsp. *michiganensis* cells (5.1 CFU/ml), IMS followed by plating on YPGA consistently detected their presence.

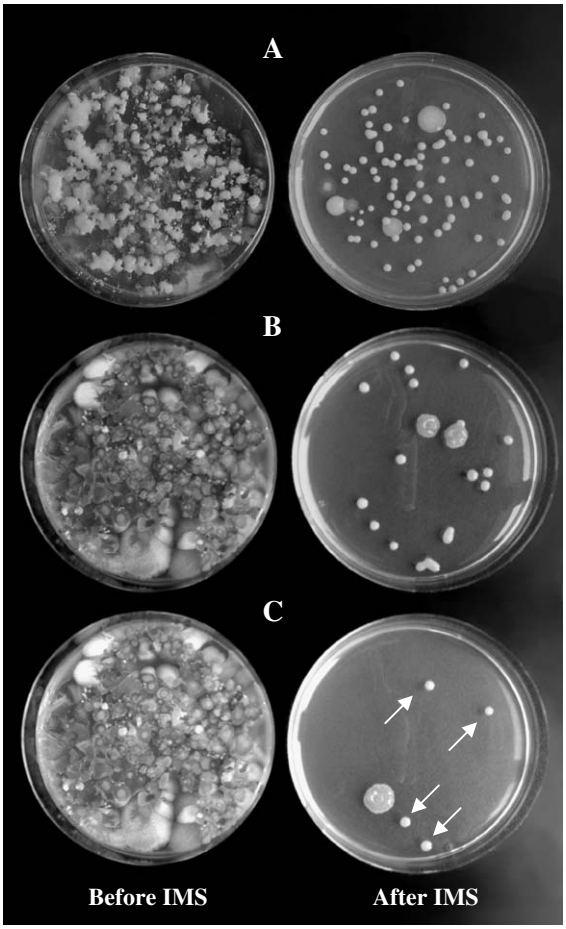

Fig. 1. Bacterial growth after 4 days of incubation in YPGA plates spread with seed extracts before and after IMS. Seed extracts contained natural saprophytic microbiota and 8.3×10^2 (A), 5.1×10 (B) and 5.1 (C) CFU/ml of *C. michiganensis* subsp. *michiganensis*. Target pathogen colonies (arrows, only indicated in C) were easily visible after IMS due to removal of most contaminants from sample.

Table 5
Detection of *C. michiganensis* subsp. *michiganensis* in naturally infected seeds by plating undiluted (1/1) and diluted (1/100 and 1/10,000) seed extracts on the non-selective medium YPGA, semiselective medium mSCM and YPGA after IMS

C. michiganensis subsp. *michiganensis* detection assay	Seed extract dilution					
	1/1	1/100	1/10,000	1/1	1/100	1/10,000
	After 4 days of incubation[a]			After 15 days of incubation[a]		
YPGA	0/4	0/4	0/4	0/4	0/4	0/4
mSCM	0/4	0/4	0/4	4/4	4/4	2/4
IMS + YPGA	4/4	4/4	4/4	4/4	4/4	4/4

The identity of suspected *C. michiganensis* subsp. *michiganensis* colonies was confirmed by NCM-ELISA and by standard tests.
[a] Positive detections in four repetitions.

3.4. Isolation of C. michiganensis subsp. michiganensis from naturally infected seeds by IMS-plating method

The results for detection in naturally infected seeds are shown in Table 5. In all cases, the pathogen was isolated on YPGA from undiluted and diluted seed extracts previously subjected to IMS to remove non-target cells. Colonies of target bacteria were recognized easily by their morphological characteristics and confirmed by NCM-ELISA and standard test. In contrast, the presence of contaminants inhibited the formation of *C. michiganensis* subsp. *michiganensis* colonies, which were never detected when undiluted and diluted seed extracts were directly plated on YPGA, nor on the semiselective medium mSCM in two samples of the most dilute seed extract (1/10,000). The remaining samples plated on mSCM were positive but colony growth was very slow and its identity could not be confirmed until 15 days after plating.

4. Discussion

The aim of this research was to develop a highly sensitive method that could aid in *C. michiganensis* subsp. *michiganensis* detection in infected tomato seed lots. This is a relatively uniform taxon that possesses common antigenic determinants (Alvarez, 2004). This

means that one antibody generally reacts with all or nearly all of its strains. Specific antisera for *C. michiganensis* subsp. *michiganensis* are commercially available and currently used for ELISA and IF, but, to our knowledge, no IMS studies have yet been done. So, we evaluated some parameters involved in the IMS process and optimized the method for the best results on the *C. michiganensis* subsp. *michiganensis* recovery rates.

Two commercial polyclonal antibodies for this pathogen were evaluated for its use in IMS. Although both were able to bind to magnetic beads, the Pab-PRI, recommended by the EPPO as an appropriate antiserum for *C. michiganensis* subsp. *michiganensis* detection (EPPO/CABI, 2005), gave better results than Pab-BR under the assayed conditions. It is well known that polyclonal antibodies produce more unwanted cross-reactions and therefore they are less specific than monoclonal antibodies. The use of monoclonal antibodies in *C. michiganensis* subsp. *michiganensis* detection has been described (Alvarez et al., 1993), although they are not used extensively. In addition, previous research showed that IMS technique using monoclonal rather than polyclonal antibodies-coated IMBs required 100 times more beads to provide similar sensitivity (Gottschalk et al., 1999). Immunomagnetic recovery with a polyclonal antiserum against *E. carotovora* subsp. *atroseptica* was greater than with two monoclonal antibodies at higher concentration (van der Wolf et al., 1996). Probably, their lower sensitivity is a consequence of the sparsely distributed epitopes recognized on target cells and so, less stable bacterium–antibody–IMB complexes are formed (van der Wolf et al., 1996; Gottschalk et al., 1999).

The effect of different concentrations of antibody and IMB on cell separation was evaluated. According to the manufacturer's specifications, a concentration of 10^7 IMBs/ml is optimal for cell recovery. It is important to take into account that, for satisfactory recovery from samples with a high number of target cells, the IMB concentration should be high in order to reduce the cell-to-bead ratio (Favrin et al., 2001). However, in plant pathogenic analysis, the main objective is to detect the pathogen at low concentration and the assay cost per sample must be considered for routine testing in diagnostic laboratories. Our results show that 10^6 IMBs/ml (10 times lower than recommended concentration), coated with a Pab-PRI dilution of 1/3200, provides good recovery. Although IMS was not intended for quantification (Grant et al., 1998), in the above conditions the aggregation index was near 1, indicating that the bacterial population could be estimated at low concentrations.

Data from this study confirm that IMS followed by plating on YPGA medium allows detection of *C. michiganensis* subsp. *michiganensis* even in the presence of large numbers of non-target bacteria and seed debris, which could significantly reduce cell recovery by IMS. It is possible that debris may provide more surface area for cell binding, effectively reducing the number of bacterial CFU available for immunocapture, and also trapping IMBs thus preventing magnetic recovery (Walcott and Gitaitis, 2000). In our case, the recovery threshold was lower than 10 CFU/ml, both in pure cultures and in the presence of saprophytes and seed debris, consistently demonstrating the high sensitivity of the method.

The detection limits reported for other techniques for *C. michiganensis* subsp. *michiganensis* are lower: 10^4 cells/ml were necessary for a positive ELISA reaction with pure cultures (Gitaitis et al., 1991) and the PCR diagnosis protocol using *C. michiganensis* subsp. *michiganensis* primers developed by Dreier et al. (1995) detected 10^2 cells/ml. To our knowledge, there are not many other techniques developed in the recent years for its detection and the most sensitive procedure is still isolation of the microorganism on semiselective media such as SCM, which detects a single contaminated seed containing 50 CFU in samples of 10,000 seeds (Fatmi and Schaad, 1988). This procedure has been proposed for the diagnosis of this bacterium by the European and Mediterranean Plant Protection Organization (EPPO) and the International Seed Health Initiative-Vegetables (ISHI). Consequently, according to these diagnostic protocols, *C. michiganensis* subsp. *michiganensis* must be isolated from plant material, including seeds, to provide a positive diagnosis (EPPO/CABI, 2005; ISHI Manual of seed health testing methods, 2005). Rapid tests like ELISA, IF or PCR can be used to identify suspected colonies after purification and for presumptive diagnosis, but plating on general nutrient and semi-selective media is necessary to obtain culturable cells for pathogenicity test. Isolation on nutrient agar can be difficult since this bacterium grows slowly, and primary isolation on YPGA takes about 96 h while saprophytes grow in less than 48 h. Even more time, up to 15 days, is necessary for its growth on selective media such as SCM, mSCM, CNS or D2ANX (Gleason et al., 1993). So the appearance of the target colonies could be inhibited preventing its detection. Furthermore, these media require a long time for preparation and require use of antibiotics that increase costs.

Our results with naturally infected seeds showed that *C. michiganensis* subsp. *michiganensis* could not be isolated by plating seed extract directly on YPGA when the saprophytic population was high. After IMS, the number of contaminants was reduced considerably and, although saprophytic bacterial colonies were still present, typical target colonies could easily be detected on YPGA and subcultured for later confirmation. The sensitivity of the method was even better than with the semiselective mSCM medium, with an additional advantage that the incubation period was considerably reduced.

In summary, IMS-plating is a fast simple method that integrates several techniques, and allows isolation of *C. michiganensis* subsp. *michiganensis* from heterogeneous seed mixtures on non-selective medium. It is highly sensitive, does not need expensive equipment nor trained personnel, and provides viable bacteria much faster than using semiselective medium. Although additional studies have to be performed for a more extensive evaluation of the method, IMS is suitable for routine testing and could be an advantageous alternative within the diagnostic protocols for *C. michiganensis* subsp. *michiganensis* in seed lots.

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