Validation Report
Clavibacter michiganensis michiganensis - Dilution plating

Validation Report of the isolation method for *Clavibacter michiganensis michiganensis*

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Abstract

In this validation report the performance indicators for the detection of *Clavibacter michiganensis subsp. michiganensis* (Cmm) in seeds of *Solanum lycopersicum* using dilution plating were determined. The validation was performed for two newly developed semi-selective media: FSCM and CMM1. The following performance indicators were determined: analytical sensitivity, specificity, repeatability, reproducibility, and robustness.

Analytical sensitivity included measuring range and detection limit. In order to determine the analytical sensitivity, tomato seed extracts with high and low saprophytic backgrounds were used. For both saprophytic backgrounds, the detection limit for Cmm was found to be 25 cfu/ml seed extract. The measuring range was found to be between 25 to at least 1.12 * 10^6 cfu/ml. Detection limit actually represents here the lower limit of the measuring range.

To determine the specificity, 20 Cmm isolates and 20 antagonistic bacteria were tested for their ability to grow on FSCM and CMM1. In total, 19 out of 20 Cmm isolates grew well on at least one of the two media. In general, Cmm isolates grew better on the new semi-selective media than the antagonistic bacteria. However, these experiments were not performed in the presence of seed extracts.

Repeatability and reproducibility were determined by testing two sets of seed samples: one with a low and one with a high infection level. The repeatability was found to be 100%. The reproducibility was found to be 94%.

Robustness experiments showed that neither variation of temperature conditions (ice vs. room temperature) at which seed extracts are kept after grinding, nor variation in incubation time had influence on the outcome of the analysis. Comparison of the new semi-selective media (FSCM and CMM1) with the old ones (SCM and D2ANX) showed that improvement in sensitivity and specificity was achieved when the new media were used. Extension of the incubation time from seven to ten days for FSCM had no influence on the outcome of the analysis. However, extension of the incubation time from seven to ten days for CMM1 led to a 5% increase in the detection of Cmm in undiluted and tenfold diluted samples.

Contents

1. Introduction

Clavibacter michiganensis subs. michiganensis (Cmm) is a bacterial pathogen with a IIAII quarantine status in seeds and propagation material of *Solanum lycopersicum* L. (tomato) intended for commercial planting. Detection of the pathogen can be done by dilution plating on semi-selective (enrichment) media or by immune fluorescence microscopy.

Detection of Cmm using semi-selective (enrichment) media is a well-known method and it has been previously described in literature (Alvarez et al., 1999; Fatmi & Schaad, 1988). This validation report contains the performance indicators for the dilution plating method which has recently developed at Naktuinbouw, using two new semi-selective media: CMM1 and FSCM. The scope applied on this validation was the detection of Cmm in seeds of *S. lycopersicum* using dilution plating. The measuring quantity is the presence of characteristic Cmm colonies on one or both of the semi-selective media. The following performance indicators were determined: analytical sensitivity, specificity, repeatability, reproducibility and robustness. The performance indicator selectivity is not relevant in this validation, since it deals with only one matrix (seeds of *S. lycopersicum)*. Validation has been performed using the documents: *Explanatory document for the validation of detection methods for plant pathogens and pests* (Van der Vlugt et al., 2007) and *the NRL-guidelines for validation en verification of methods version 1.0* (Dutch Plant Protection Organisation, 2008). The experiments have been performed between August and December 2009.

2. Analytical sensitivity

2.1 Introduction

The sensitivity of an analysis can be defined as *the limits in which the analysis can be applied reliably*. The lower bound corresponds with the detection limit. This is *the lowest concentration of the component in a laboratory sample of which the presence can be determined reliably.*

2.2 Material & methods

Preparation of the seed samples:
In order to determine the analytical sensitivity, seed lots of *S. lycopersicum* with two different saprophytic backgrounds were used: one with a high (seed lot *ZZB53*) and one with a low saprophytic background (seed lot *ZZB53 autoclaved*). In order to create a high saprophytic background, 5000 seeds of *ZZB53*, which were initially autoclaved, were mixed with 20 seeds of *ZZB53*. For the low saprophytic background, 5000 seeds of *ZZB53* were used after being autoclaved. In order to establish the actual concentration of the present saprophytic micro flora a pilot experiment was performed. It was found that 100 µl seed extract resulted in about 150 saprophytic bacteria for the high and 15 saprophytic bacteria for the low saprophytic background on CMM1, respectively (data not shown). For each background, eight samples were processed following the standard protocol of Naktuinbouw (SPN-B004, of 15/01/2010) until the concentration step (step 5). At this stage, the concentrated sub samples of each saprophytic background were pooled to one concentrated seed extract. These two concentrated seed extracts (one with high and one with low saprophytic background) were used to determine the measuring range and the detection limit.

Measuring range and detection limit:
1 ml of each concentrated seed extract (for both the high as well as the low saprophytic background) was spiked with Cmm (reference strain *zumm3095*) to a final concentration of $1.12 * 10^7$ cfu/ml. A tenfold dilution series was made until a theoretical Cmm concentration of 0.01 cfu/ml seed extract. All individual dilutions were handled as normal “extracts” and tested according to the standard protocol (SPN-B004, of 15/01/2010) of Naktuinbouw. The identity of suspected colonies, grown on either FSCM or Cmm1, was confirmed by checking their characteristics after being transferred on YDC and GF with rifampicine.
The experiment was repeated three times. The detection limit was calculated as the average of the lowest Cmm concentration detected in each experiment plus three times the standard deviation.

2.3 Results

Measuring range and detection limit were both determined by making two series of tenfold dilutions of Cmm directly in seed extracts (one series for the high and the second for the low concentration of saprophytic bacteria). In cases of low concentrations of saprophytic bacteria present in the seed extract, Cmm could be successfully detected at a minimum concentration of 11.2 cfu/ml seed extract. This was the case in two of the three experiments. However, in the third experiment Cmm could be successfully detected at even a concentration of 1.12 CFU Cmm/ml seed extract (table 1). Based on the data, related to the seed extract with a low

concentration of saprophytic micro flora, it has been concluded that the detection limit of Cmm was found to be 25 CFU/ml extract.

Similar results were found in the cases of seed extracts containing a high concentration of saprophytic bacteria. In the first two experiments, Cmm could be successfully detected at a minimum concentration of 11.2 cfu/ml seed extract. However, in the third experiment Cmm could be successfully detected at even a concentration of 1.12 CFU Cmm/ml seed extract (table 2). Based on the data related to the seed extract with a high concentration of saprophytic micro flora, it has been concluded that the detection limit of Cmm was determined to be 25 CFU/ml seed extract.

Table 1: Dilution series of Cmm in seed extracts containing a low concentration of saprophytic bacteria for the determination of the measuring range and detection limit. + = detected, - = not detected, n.t. = not tested.

Cmm	Experiment 1		Experiment 2		Experiment 3	
(CFU /ml)	FSCM	Cmm1	FSCM	Cmm1	FSCM	Cmm1
$1{,}12 * 10^{6}$	+	+	n.t.	n.t.	n.t.	n.t.
$1{,}12 * 10^{5}$	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
$1{,}12 * 10^{4}$	+	+	n.t.	n.t.	n.t.	n.t.
$1{,}12 * 10^{3}$	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
$1{,}12 * 10^{2}$	+	+	+	+	+	+
$1{,}12 * 10^{1}$	+	+	-	+	+	+
$1{,}12 * 10^{0}$	-	-	-	-	+	-
$1{,}12 * 10^{-1}$	+	-	-	-	-	-
$1{,}12 * 10^{-2}$	-	-	-	-	-	-
nc	-	-	-	-	-	-

Table 2: Dilution series of Cmm in seed extracts containing a high concentration of saprophytic bacteria for the determination of the measuring range and detection limit. . + = detected, - = not detected, n.t. = not tested.

Cmm	Experiment 1		Experiment 2		Experiment 3	
(CFU /ml)	FSCM	Cmm1	FSCM	Cmm1	FSCM	Cmm1
$1{,}12 * 10^{6}$	+	+	n.t.	n.t.	n.t.	n.t.
$1{,}12 * 10^{5}$	n.t.	n.t.	n.t.	n.t.	n.t.	n.t.
$1{,}12 * 10^{4}$	+	+	+	+	+	+
$1{,}12 * 10^{3}$	n.t.	n.t.	+	+	+	+
$1{,}12 * 10^{2}$	+	-	+	+	+	-
$1{,}12 * 10^{1}$	+	+	+	-	+	-
$1{,}12 * 10^{0}$	-	-	-	-	+	-
$1{,}12 * 10^{-1}$	-	-	-	-	-	-
$1{,}12 * 10^{-2}$	-	-	n.t.	n.t.	n.t.	n.t.
nc	-	-	-	-	-	-

2.4 Discussion

Results obtained from the analytical sensitivity experiments on the detection of Cmm using dilution plating on the two new semi-selective media revealed that the detection limit for Cmm is at 25 cfu/ml seed extract. This was found to be true for both seed extracts (high as well as low saprophytic background). Compared to other validated dilution methods (*Ralstonia solanacearum* validated at the Dutch Plant Protection service), this method is very sensitive and within an acceptable range.
Because the detection limit actually represents the lowest limit of the measuring range, the analysis is able to detect Cmm at concentrations varying between 25 and at least $1.12 * 10^6$ CFU Cmm/ml seed extract,. In practice this means that it is unlikely that Cmm infected samples would not be detected due to a concentration of Cmm which is not within the measuring range of the analysis.

Based on the results, both the measuring range as well as the detection limit, are found to be acceptable.

3. Specificity

3.1 Introduction

The specificity of an analysis can be defined as *the ability of a detection method to distinguish the pathogen from other (related) organisms and the degree of the analysis to distinguish variants of the target organism.* To determine the specificity of an analysis different isolates of the target organism should be tested to determine whether the analysis is able to detect these isolates. Furthermore, potential cross-reactions with other organisms should be assessed.

3.2 Material & methods

Isolates**:**
Based on a phylogenetic tree generated at Naktuinbouw using the AFLP patterns of more than 500 Cmm isolates, a selection of 20 Cmm isolates was made. The selected Cmm isolates are known to be genetically distant and have previously been assessed as positive in a bioassay. Furthermore, antagonistic bacteria, that are frequently present on seeds of *S. lycopersicum* have been previously characterized based on 16s rDNA sequence analysis. Among these antagonistic bacteria, twenty isolates covering eight genera were selected for assessing the specificity of the isolation method of Cmm on the new semi-selective media (table 3).

Table 3: Cmm, related species and their corresponding isolate numbers used for determination of specificity.

Species	Isolate number (Naktuinbouw)	Total
Acinetobacter sp.	40	1
Aerococcus sp.	33	1
Aerococcus viridans	34	1
Clavibacter michiganensis subsp. michiganensis	10, 11, 14, 15, 19, 20, 23, 24, 25, 56, 59, 60, 62, 86, 87, 96, 277, 279, 280, 281	20
Curtobacterium spp.	38, 42, 44, 45, 46, 47	6
Enterococcus faecalis	3	1
Lactococcus lactis	26, 32, 36	3
Leuconostoc spp.	8, 11, 39,	3
Microbacterium spp.	35, 37, 41, 43	4

Droplet method:
Bacterial suspensions at an approximate concentration of 10^9 cfu/ml were made in 0.07 M phosphate buffer for all the isolates (Table 3). For each isolate (Cmm or antagonist) a tenfold dilution series was made and a 5 µl droplet of the dilutions 10^4 to 10^7 were put on CMM1, FSCM and GF as a control, using a multichannel pipette. With the scoring system given in *table 4*, each isolate was scored for being present/absent in the different dilutions for each of the above mentioned media. Based on this scoring system, a high score (minimum of 3) is required on at least one of the two semi-selective media for Cmm isolates, whereas for isolates belonging to the antagonistic bacteria a low score is preferably acquired.

Table 4: Scoring system for the droplet method.

Dilution	Bacteria p	Score
-	-	0
10^4	+	1
10^5	+	2
10^6	+	3
10^7	+	4

3.3 Results

The ability to grow on CMM1 and FSCM was evaluated for 20 Cmm isolates and 20 antagonistic bacteria. Using the droplet method, all Cmm-isolates scored a minimum of three points on one or both of the selective media, with the exception of isolate number 60 (figure 1).

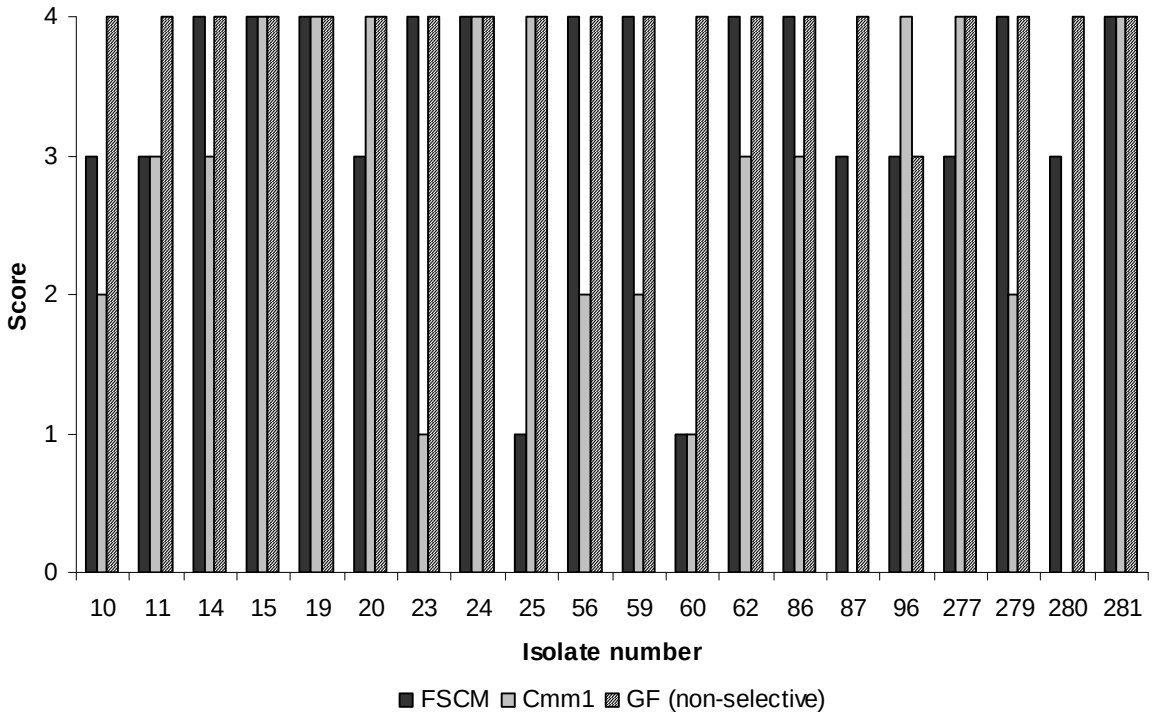

Figure 1: Individual scores acquired by the droplet method for the 20 Cmm isolates grown on the three different media.

In order to compare the growth of the Cmm isolates with the growth of the antagonistic bacteria, all the individual scores acquired from the Cmm isolates were added together and compared with the cumulative score of the antagonistic bacteria. In *figure 2* the cumulative scores are shown for Cmm and the antagonistic bacteria per medium. Differences in cumulative score were found for Cmm versus antagonistic bacteria on FSCM (67 versus 33) and CMM1 (54 versus 28) media. This difference in cumulative score was absent on the non-selective GF medium (79 versus 70).

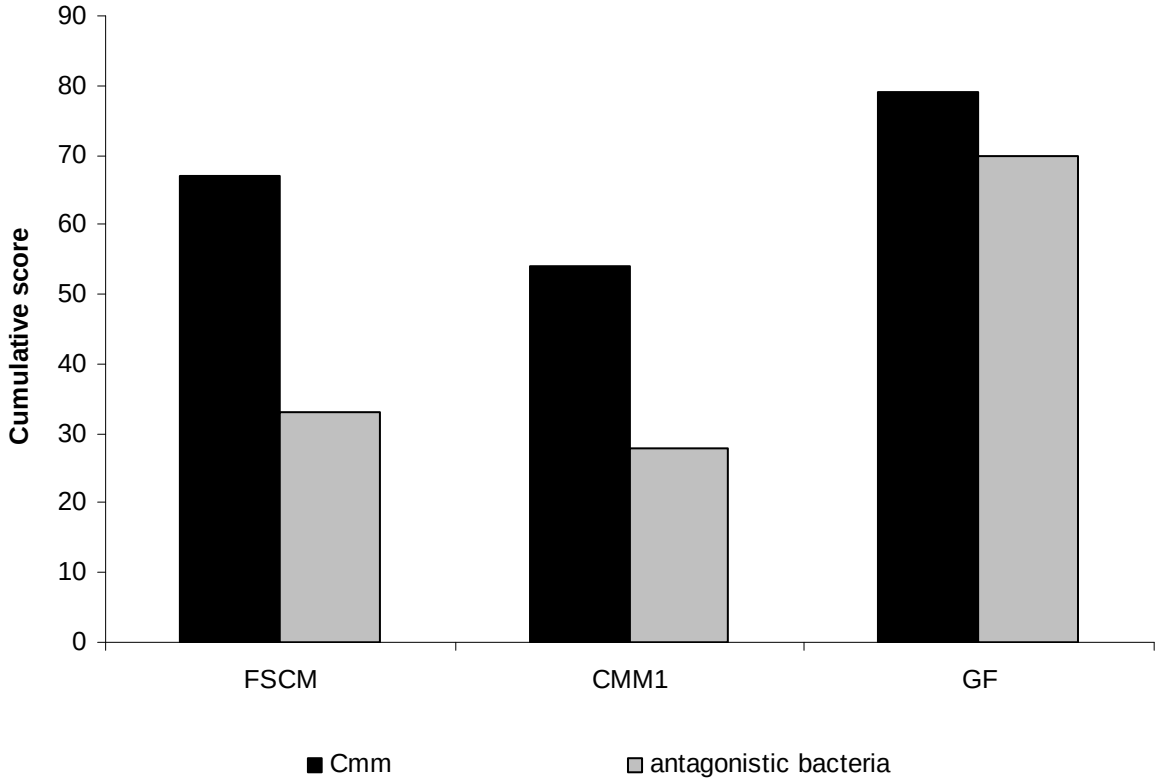

Figure 2: Cumulative score of the droplet method shown per medium for Cmm and the antagonistic bacteria.

3.4 Discussion

The specificity was determined by testing the ability of different Cmm isolates and known antagonistic bacteria to grow on FSCM and CMM1. With the exception of Cmm isolate number 60, all the Cmm isolates performed well (scored 3 or higher) in terms of growth on the selective media. Further examination of Cmm isolate 60 revealed that morphology of Cmm 60 was atypical, but it was able to growth on the conventional media (SCM and D2ANX).

Comparison of the growth performance of Cmm with the antagonistic bacteria showed that in general the Cmm isolates were able to growth better on the selective media than the antagonistic bacteria.

4. Repeatability & Reproducibility

4.1 Introduction

The repeatability can be defined as *the conformity between results of successive measurements, which are obtained under the same circumstances by the same person in a laboratory within a certain time.* The reproducibility can be defined as *the conformity between results of measurements of the same measuring quantity, obtained under different, specified circumstances by different persons in a laboratory.*
Repeatability and reproducibility should be determined to obtain information about the sensitivity of the analysis for small variation in the execution under routine like circumstances.

4.2 Material & methods

Preparation of the samples:
For the preparation of the seed samples, *S. lycopersicum* seeds artificially infected with Cmm (isolate 277), were used (seed lot a and b; table 5). This seed lot was previously used in a ring test and was scored positive by all participants. Samples were prepared by combining seed lots as given in *table 5*. In order to create samples with a high Cmm infection level, 20 seeds of seed lot *c* and 25 seeds of seed lot *a* were added to 4955 seeds of seed lot *d*. In order to create a low Cmm infected sample, 150 seeds of seed lot *b* and 20 seeds of seed lot *c* were added to 4830 seeds of seed lot *d.* For each infection level 16 subsamples were created.

Table 5: Properties of the used seed lots for the preparation of the seed samples.

Seedlot	origine	properties	treatment
a	Nakt ZZB 17	Few saprophytic bacteria	spiked with 2,0 * 10^6 cfu/ml Cmm
b	Nakt ZZB 17	Few saprophytic bacteria	spiked with 2,0 * 10^4 cfu/ml Cmm
c	Nakt ZZB 53	Lots of saprophytic bacteria	-
d	Nakt ZZB 53	Lots of saprophytic bacteria	autoclaved

Experimental setup:
The experiments regarding repeatability and reproducibility were determined using the experimental setup of the NRL-guidelines (table 6). For repeatability, eight sub samples for the high and eight sub samples for the low Cmm infection level, were tested at one time point, by the same person, using the same equipment. For repeatability, a similar setup was used with the difference that the sub samples were tested at eight different time points, by different persons. Suspected colonies were confirmed by real-time PCR (data not shown). Repeatability and reproducibility were calculated as the percentage of samples which were found positive for Cmm.

Table 6: Experimental setup for the determination of the repeatability and reproducibility.

Cmm	Repeatability	Reproducibility							
	Timepoint 1	Timepoint 2	Timepoint 3	Timepoint 4	Timepoint 5	Timepoint 6	Timepoint 7	Timepoint 8	Timepoint 9
high	xxxxxxxx	x	x	x	x	x	x	x	x
low	xxxxxxxx	x	x	x	x	x	x	x	x

4.3 Results

In order to determine repeatability and reproducibility eight sub samples with a high Cmm infection level and eight sub samples with a low infection level were analyzed using the experimental setup in *table 6*. For repeatability, Cmm was detected in all 16 samples. For reproducibility in one sample (*sample set 2; low infection level*) Cmm could not be detected in any of the two media used. Repeatability was found to be 100% and reproducibility was found to be 93,8%.

Table 7: Results for repeatability and reproducibility.

Repeatability				Reproducibility			
sample set	Infection level	FSCM	CMM1	sample set	Infection level	FSCM	CMM1
1	low	+	+	1	low	+	-
	high	+	+		high	+	-
2	low	+	+	2	low	-	-
	high	+	+		high	+	-
3	low	+	+	3	low	+	-
	high	+	+		high	+	+
4	low	+	+	4	low	+	+
	high	+	+		high	+	-
5	low	+	+	5	low	+	+
	high	+	+		high	+	-
6	low	+	+	6	low	+	-
	high	+	+		high	+	-
7	low	+	+	7	low	+	-
	high	+	+		high	+	+
8	low	+	+	8	low	+	-
	high	+	+		high	+	+

4.4 Discussion

Repeatability and reproducibility provide information about how the analysis is able to cope with small variations, which can occur under routine like circumstances. Repeatability can be seen as the minimum variation one could expect from the analysis. Here, the repeatability was found to be 100 percent. For reproducibility one of the sub samples of the Cmm low infection level was not detected. A second evaluation of the semi-selective media for this sub sample showed that Cmm was present, but probably not detected because not enough suspected colonies were transferred to YDC. Thus, it is very important to select and transfer enough suspected Cmm colonies. In all other samples Cmm was detected, therefore the reproducibility was found to be 94 percent. The results found for repeatability and reproducibility show that the analysis is able to detect Cmm under varying circumstances.

5. Robustness

5.1 Introduction

The robustness can be defined as *the degree of insensitiveness of the measuring result for deviations in execution, circumstances and quality of the used materials.* In practise the robustness is determined by deviation of the most important parameters, which can have an impact on the outcome of the analysis.

Under routine-like circumstances, it often happens that a lot of samples have to be processed simultaneously. After the seed extract has been prepared it is put on ice until further processing. By keeping the temperature low, the interaction and growth of bacteria in the seed extract is minimized. The importance of minimizing the interaction of bacteria was tested by comparison of seed extracts, which were kept on ice and seed extracts which were kept at room temperature.

Selection of semi-selective media, which are used in the analysis, is one of the most critical components of the test. Therefore, the performance of semi-selective media FSCM, CMM1, SCM and D2ANX was compared to see how the new semi-selective media perform compared to the old ones.

Incubation time on the old media SCM and D2ANX is ten days. For the new semi-selective media FSCM and CMM1, the incubation time is reduced to seven days. The influence of the incubation time was tested by evaluating all four media after both seven and ten days of incubation.

Summarizing, we defined temperature, where the concentrated seed extracts are kept, incubation time on the semi-selective medium and performance of the old and new semi-selective media important parameters which could influence the outcome of the analysis.

5.2 Material & methods

Temperature of the seed extracts:
Eight seed samples were prepared by adding 20 seeds with a high saprophytic background (*ZZB53*) to 5000 “clean” seeds (*ZZB53 autoclaved*). The samples were processed according to the standard protocol of Naktuinbouw (SPN-B004, of 15/01/2010) until the concentration step. At this stage, each sample was spiked with Cmm (reference strain *zumm3095*) to a final concentration of 1120 cfu/ml seed extract and divided into two sub samples. Of each sample, one sub sample was kept on ice whereas the other one was kept at room temperature for four hours. Subsequently, all the sub samples were plated on the semi-selective media, incubated and evaluated according to the standard protocol of Naktuinbouw (SPN-B004, of 15/01/2010). The identity of suspected colonies, grown on either FSCM or Cmm1, was confirmed by checking their characteristics after being transferred on YDC and GF with rifampicine.

Comparison of semi selective media (performance and incubation time):
In order to compare the performance of the semi-selective media, six non-infected seed lots from routine testing (*15, 1862, p46.010, p46.011, 1825, 1816)* and one naturally Cmm infected seed lot *(ZZB 391)*, were selected. Of each seed lot three sub samples of 5000 seeds

were taken. All dilutions (0x, 10x, 100x and 1000x) of the non-infected seed lots, were spiked with Cmm (reference strain *zumm3095*) to a final concentration of 1120 CFU/ml seed extract. All seed extracts were plated on FSCM, CMM1, D2ANX and SCM. The semi-selective media were evaluated after seven and after ten days of incubation, by scoring the most concentrated dilution of a sub sample in which Cmm could be detected. The identity of suspected colonies, grown on the different semi-selective media, was confirmed by checking their characteristics after being transferred on YDC and GF with rifampicine.

5.3 Results

The influence of the temperature, at which concentrated seed extracts are kept, was tested by incubating one sub sample on ice, whereas the second subsample of the same sample was incubated at room temperature. In all sub samples tested, both on ice and at RT, Cmm could be detected. For the semi-selective medium FSCM, significantly more suspected CMM colonies were observed, in sub samples kept at RT, than in those kept on ice (paired t-test; $p \leq 0.05$) (figure 3). Although the same trend was observed for the semi-selective medium Cmm1, no significant difference in numbers of suspected Cmm colonies were observed in the sub samples incubated on ice and at RT (figure 4).

Comparison of the total number of suspected Cmm colonies with the total number of all bacterial colonies found in all the eight samples among the two treatments (incubation on ice or at RT) showed that there was little difference between these treatments. For the semi-selective medium FSCM the percentage Cmm colonies after incubation of the seed extract on ice and at RT was 0.76 and 0.80, respectively. For CMM1 this percentage was remarkably lower (0.29 and.0.23, respectively) (figure 5).

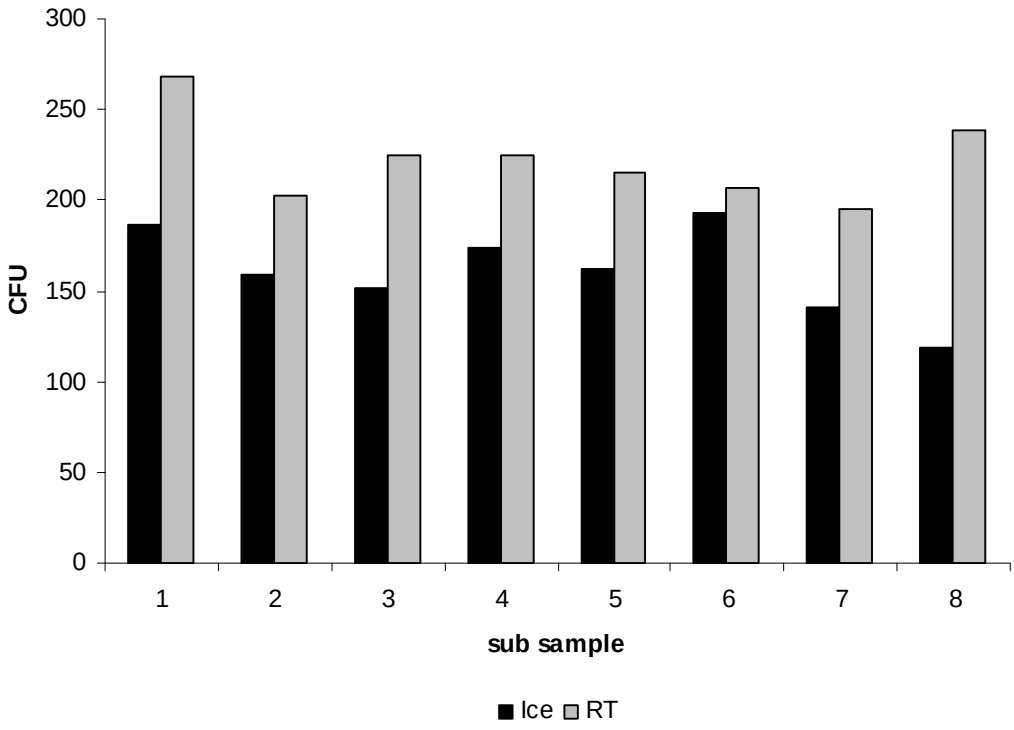

Figure 3: Number of suspected Cmm colonies detected after plating the eight samples on the semi-selective medium FSCM. Samples (concentrated extracts) were incubated on ice or at room temperature (RT). Incubation on ice led to significantly more suspected Cmm colonies (paired t-test $p \leq 0.05$).

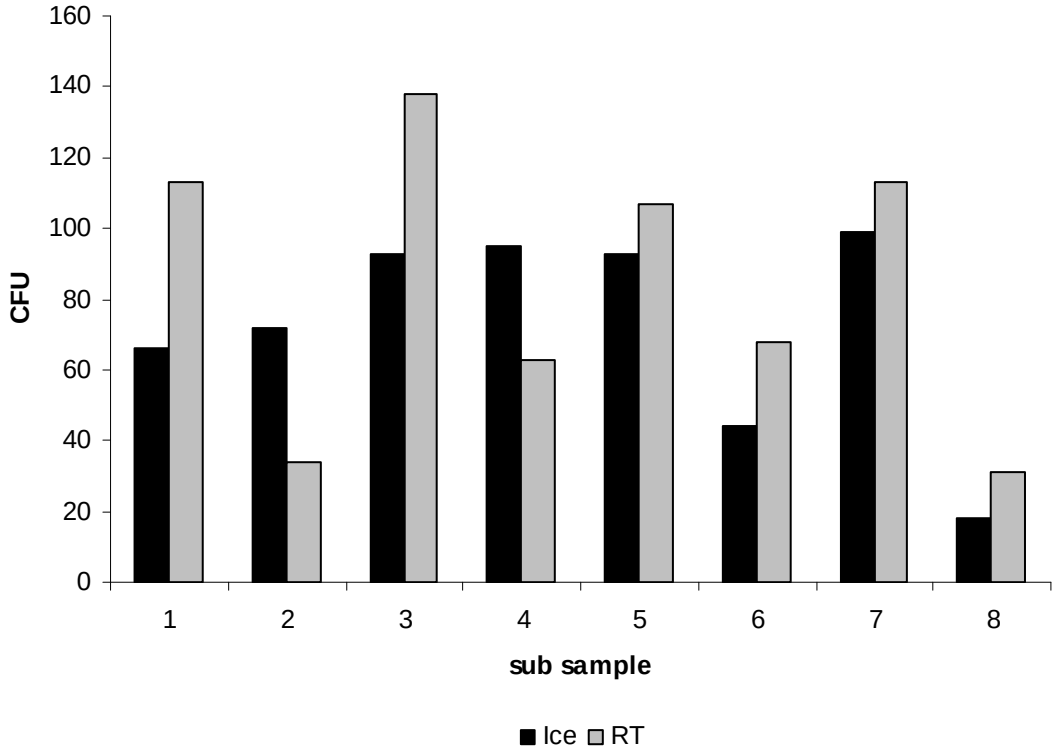

Figure 4: Number of suspected Cmm colonies detected after plating the eight samples on the semi-selective medium Cmm1. Samples (concentrated extracts) were incubated on ice or at room temperature (RT). No significant differences were found between the incubation on ice and at RT

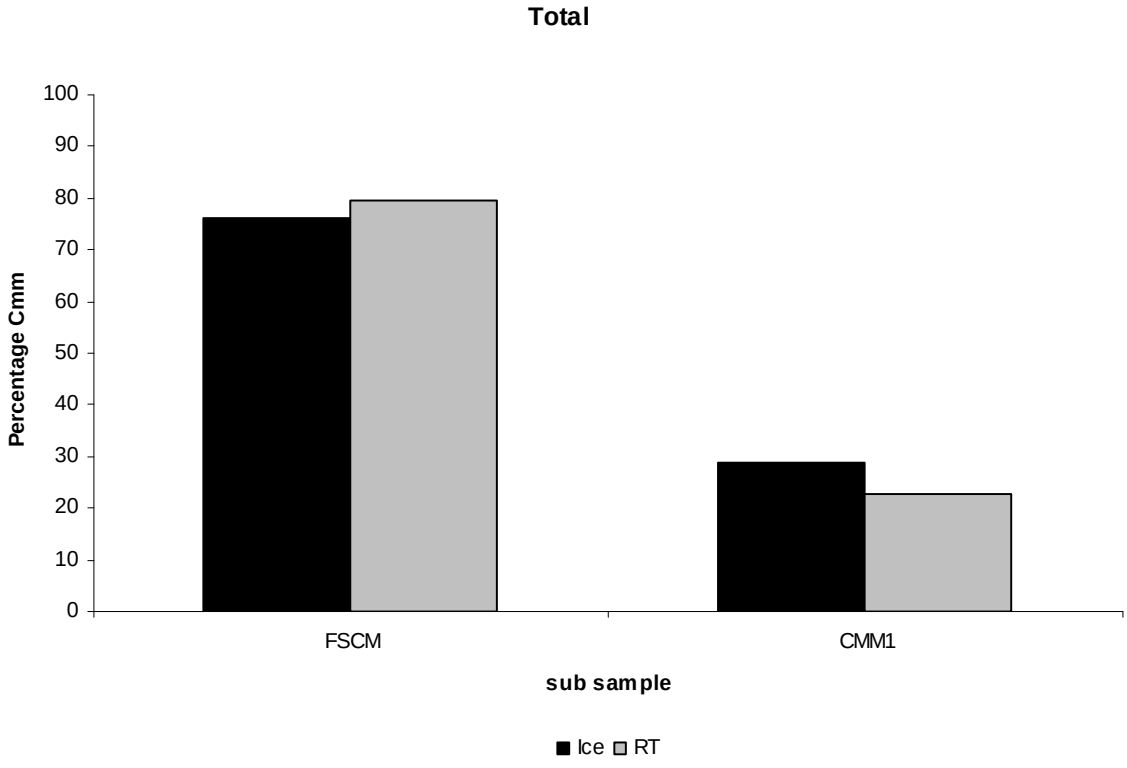

Figure 5: Percentage of suspected Cmm colonies compared to the total observed colonies in the eight samples, incubated on ice or at room temperature (RT).

Comparisons were performed between the semi-selective media FSCM, CMM1, SCM and D2ANX by isolating Cmm from 7 different seed lots originating from different origins. After seven days of incubation, the semi-selective medium FSCM gave the best results regarding growth of Cmm. From the 21 seed samples tested, Cmm could be successfully detected in 86% of the cases in the undiluted seed extract, whereas 14% in the tenfold dilution. After seven days of incubation, the semi-selective medium CMM1 performed well. Although Cmm could be detected in both undiluted and in the tenfold dilution at comparable levels to SCM (71% vs. 71%), Cmm could successfully be detected in 20 out of 21 seed samples plated on Cmm1 in the undiluted, tenfold and hundredfold dilution, which was not the case with the semi-selective medium SCM. The semi-selective medium D2ANX performed the worst: Cmm could be detected in both the undiluted and the tenfold dilution at 43% of the seed samples. Furthermore, in 28% of the seed samples detection of Cmm could not be realized at all (figure 6).

After ten days of incubation, similar results were found for the detection of Cmm on the three semi-selective media FSCM, CMM1 and SCM. CMM1 showed a 5% increase in detection of Cmm in the undiluted and tenfold diluted samples. However, D2ANX showed an increase in detection of Cmm in the undiluted and tenfold dilution of 14%. Furthermore there was a large reduction in the number of seed samples, from 28% to 10%, in which Cmm could not be detected at all (figure 7).

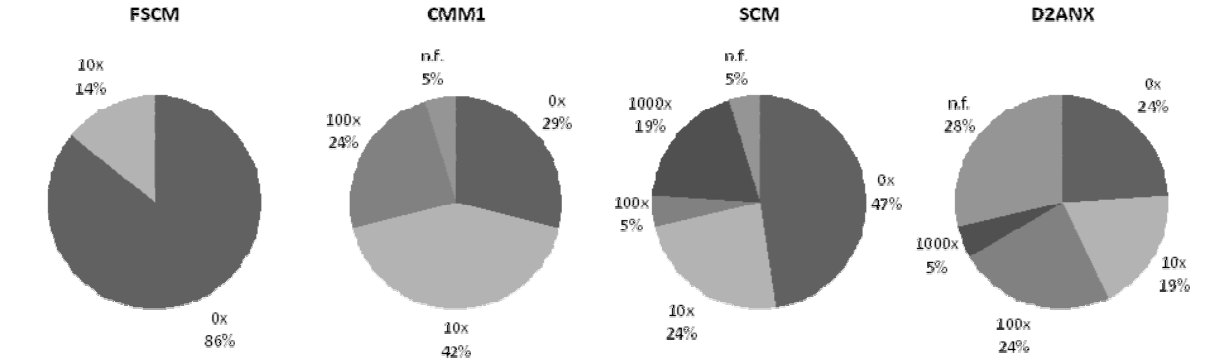

Figure 6: Distribution of the least diluted samples in which Cmm could be detected after plating on semi-selective media FSCM, CMM1, SCM and D2ANX after seven days of incubation. N.f. = Cmm not found.

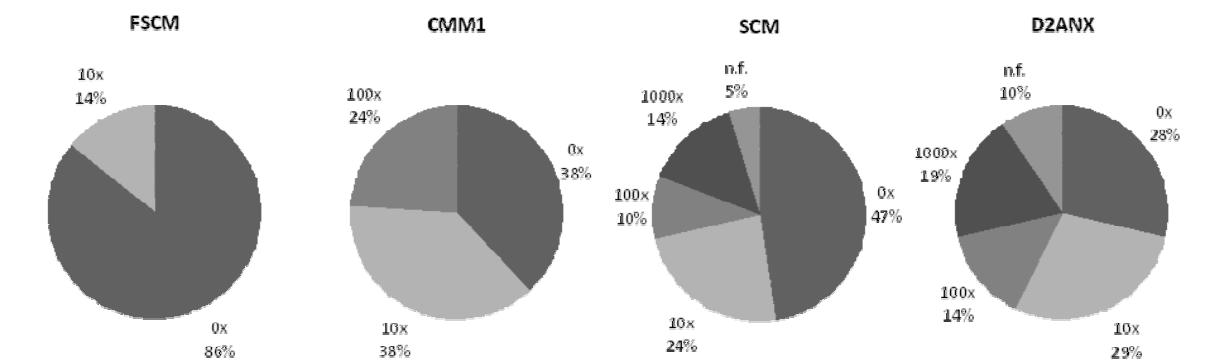

Figure 7: Distribution of the least diluted samples in which Cmm could be detected after plating on semi-selective media FSCM, CMM1, SCM and D2ANX after ten days of incubation. N.f. = Cmm not found.

5.4 Discussion

The robustness was determined by evaluating:
i. The effect of temperature on the seed extracts, ii. The performance of the new semi-selective media against the old ones and iii. The effect of the incubation time on the different media
Concentrated seed samples spiked with Cmm, which were kept at room temperature, showed a significantly increased recovery of Cmm on FSCM compared to seed extracts, which were kept on ice. For CMM1 the results were less consistent and probably due to large variations in number of suspected colonies, no significant effect was observed. This can be explained due to the fact that not only the recovery of Cmm was increased but also the recovery of saprophytic bacteria. Especially on CMM1 which is less specific for Cmm compared to FSCM. This is in accordance with other experiments were often Cmm could not be found in seed samples containing high numbers of saprophytic bacteria.

Comparison of the performance of the semi-selective media FSCM, CMM1, SCM and D2ANX after seven and ten days of incubation, showed that the new media (FSCM and CMM1) perform better compared to SCM and D2ANX. With FSCM and CMM1, Cmm was detected in all of the tested seed samples and most of the time this was in the undiluted or tenfold dilution of the seed extract. This in contrast to SCM and D2ANX were (after ten days) of incubation Cmm could not be detected at all in 5% and 10% of the seed samples.

The effect of the incubation time was minimal for the new semi-selective media: FSCM and CMM1 gave similar results after seven or ten days of incubation. However, for SCM, and especially for D2ANX, incubation time is a very crucial parameter as incubation for at least ten days decreases the chance to miss Cmm.

6. General conclusions

In this validation report the performance indicators are determined for the detection of Cmm in seeds of *S. lycopersicum* using dilution plating on two new semi-selective media FSCM and CMM1. The measuring quantity is the presence of characteristic Cmm colonies on the selective enrichment media FSCM and/or CMM1.

Based on results acquired from the analytical sensitivity and specificity, the analysis is sensitive enough for its intended purpose and was able to detect all tested Cmm isolates with the exception of one isolate.

Based on results acquired from repeatability and reproducibility, it can be concluded that the method is not susceptible for small variations which can occur under routine-like circumstances. Finally, robustness showed that incubation of seed extracts at RT (within reasonable time constraint) has no influence on the outcome of the analysis. Seven days of incubation on the media was found to be sufficient. Extending the incubation time makes no difference for FSCM and only a small difference for CMM1. Comparison of Cmm detection between the new semi-selective media FSCM, CMM1 and the old ones SCM, D2ANX, showed that the sensitivity of the new media is remarkably improved compared to the old ones.

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