CLAVIBACTER MICHIGANENSIS SUBSP. *MICHIGANENSIS* – CHECKING THE SENSITIVITY OF THE INDIRECT IMMUNOFLUORESCENCE METHOD

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ABSTRACT

Clavibacter michiganensis subsp. michiganensis is a Gram positive actinobacteria, which attacks tomatoes, causing their bacterial wilt. It causes significant damage in the affected regions, unless detected at time and no preventive and control measures are taken. Detection is carried out by different laboratory techniques, including the indirect immunofluorescence, a sensitive technique but not sufficient to confirm this harmful quarantine agent.

INTRODUCTION

Clavibacter michiganensis subsp. *michiganensis* represents the causing agent of ”Bacterial canker of tomato” or ”The disease from Grand Rapids” being a harmful organism for quarantine in many countries (Severin & Iliescu, 2006). It was for the first time isolated, by Smith, in North America, at the beginning of the 20th century (De León et al., 2011). It is widely spread on most continents (Africa, Asia, America, Europe, and Oceania), measures of preventing and fighting against it being necessary in order to avoid its spreading (https://www.cabi.org/ isc/ datasheet/15338, 2018).

As regards the taxonomy, this bacteria is part of:
Domain *Bacteria*
Filum *Actinobacteria*
Class *Actinobacteria*
Subclass *Actinobacteridae*
Order *Actinomycetales*
Suborder *Micrococcineae*
Family *Microbacteriaceae*
(https://www.cabi.org/isc/datasheet/15338).

Clavibacter michiganensis subsp. michiganensis grows and develops at temperatures between 20-30^{0}C. It has the optimal rise temperature at 25^{0}C, and the maximum survival limit at 50^{0}C (Sen et al., 2015).

Its spreading and disseminating is carried out by means of contaminated planting material, vegetable waste, infected soil, agricultural practices, contaminated equipment, water drops, aerosols, insects, wind and by contact among leaves (Rădulescu și colab., 1970; Milijašević et al., 2007; Kawaguchi et al., 2010; Xu *et al.*, 2010).

In order to be able to prevent it, laboratory tests are required to certify that planting material (seed or seedlings) are free of this harmful organism. However, if the infection has taken place, in order that the applied measures to be appropriate, must be identified the infected plants and then be tested to confirm the infection with *Clavibacter michiganensis* subsp. *michiganensis.*

The EPPO protocol “PM 7/42 (3) *Clavibacter michiganensis* subsp. *michiganensis*” recommends two test diagrams for detection and identification of this organism. Thus, if the planting material is represented by seeds, these ones undergo the bacterial extraction step by soaking or crushing, and then the obtained extracts either are directly isolated on the culture medium or firstly subjected to rapid tests (PCR and immunofluorescence), and then isolated on culture medium. Whether the planting

material is represented by plants or parts of plants with symptoms, they are subjected to the bacterial extraction by crushing and then the extract obtained is isolated on the culture medium. Whatever the situation, after obtaining bacterial colonies with morphological and cultural characters similar to the bacterium of interest, they are subjected to identification tests (serological / biochemical / molecular). If at least two tests are positive, then it is passed to the pathogenicity test to confirm *Clavibacter michiganensis* subsp. *michiganensis*.

One of the serological tests recommended by the above protocol is the indirect immunofluorescence test. This test allows the visualisation of antigen by microscope in the ultraviolet light. It uses antibodies marked with a fluorescent compound called fluoride (https://ro.wikipedia.org/wiki/Imunofluorescen%C8%9B% C4%83).

According to the EPPO protocol ”PM 7/97 (1) Indirect Immunofluorescence test for plant pathogenic bacteria”, the detection threshold of this test is of 10^3-10^4 cells/ml. In fact, this one is the minimum limit of detection (the lowest value of the bacterial concentration which can be detected), meaning the inferior limit of the practical application method. By establishing the minimum limit of detection, the sensitivity of the indirect immunofluorescence method can be determined.

The sensitivity of a method is one of the six parameters (specificity, selectivity, sensitivity, repeatability, reproductibility and robustness) used in the verification of a working method in the laboratory according to the EPPO protocol PM 7/98 (3) ”Specific requirements for laboratories preparing accreditation for a plant pest diagnostic activity”.

The present experiment aims to verify the indirect immunofluorescence method in diagnostic laboratories in Romania.

MATERIAL AND METHOD

The antiserum which are used in this test came from Loewe® Biochemica GmbH (Germany). There were used two antibodies: polyclonal antibodies ex goat (used at the dilution of 1/2000) and rabbit-anti-goat IgG (H+L) FITC (used at the dilution of 1/200). Both working dilutions are the dilutions recommended by the producer.

In order to check the sensitivity of the indirect immunofluorescence method in detecting *Clavibacter michiganensis* subsp. *michiganensis*, there were realised bacterial suspensions (with a low turbidity) coming from:

- two reference bacterial strains of *Clavibacter michiganensis* subsp. *michiganensis* (PD 223 - Hungary origin, purchased from Plant Protection Service, Wageningen, the Netherlands; NCPPB 2979 - Hungary origin, purchased from Food and Environment Research Agency Sand Hutton, York, Great Britain);
- six bacterial strains coming from tomatoes plants, previously detected, within the National Phytosanitary Laboratory (National Phytosanitary Authority, Romania), as being contaminated with *Clavibacter michiganensis* subsp. *michiganensis* (19-3857, 19-3858, 19-3861, 19-4088, 19-4326, 19-4327).

The suspensions were carried out in one ml of sterile water and for each bacterial suspension, hereinafter referred to as the mother suspension, more than one decimal dilution were performed. For a previous determination of the bacterial concentration, each dillution was pipetted in one window, on an slide with eight windows (figure 1), and afterwards it was tested. The test was done according to the protocol EPPO ”PM 7/97 (1) Indirect Immunofluorescence test for plant pathogenic bacteria”. Slides visualisation was achieved with epifluorescence miscroscope, with filters for excitation of FITC, under oil immersion, at a magnification of 1000.

RESULTS AND DISCUSSIONS

Cell count, with living fluorescence and typical morphology (figure 2) was done on microscopic field, a reading of 40 fields per window being necessary, according to EPPO protocol ”PM 7/97 (1) Indirect Immunofluorescence test for plant pathogenic bacteria”. The same protocol recommends the following formula for the determination of the bacterial concentration:

$$\mathbf{N = C \cdot 1000 / y \cdot F}$$

where:
C = number of fluorescent typical cells / window ($C = c \cdot S / s$**);**
c = medium number of bacterial cells / 40 microscopic fields;
S = well surface **(**$S = \pi R^2$**);**
R = well ray (R = 3 mm for 6 mm window or R = 4 mm for 8 mm window);
s = lens surface **(**$s = \pi i^2 \div 4G^2K^2$**);**
F = dilution factor of the extract (F = 1);
i = field coefficient (i = 22) - it depends on the used microscope;
K = tube coefficient (K = 1,25) - it depends on the used microscope;
G = lens thickness (G = 100× or G = 60× or G = 50×);
y = extract volume (y = 0,02 ml for 6 mm window or y = 0.04 ml for 8mm window)

consequently:
$\mathbf{N = c \cdot \pi \cdot R^2 \cdot 4 \cdot G^2 \cdot K^2 \div \pi \cdot i^2 \cdot y \cdot F = c \cdot R^2 \cdot 4 \cdot G^2 \cdot K^2 \div i^2 \cdot y \cdot F =} c \cdot 3^2 \cdot 4 \cdot 100^2 \cdot 1{,}25^2 \div 22^2 \cdot 0{,}02 \cdot 1 = \mathbf{c \cdot 58109{,}5}$

The results of readings by the epifluorescence microscope and the bacterial concentration of *Clavibacter michiganensis* subsp. *michiganensis* suspensions, which were used are presented in table 1.

Due to the large number of cells in some windows, cells could not be counted and the bacterial concentration could not be calculated. It was possible to count the cells and determine the bacterial concentration, by applying the above-mentioned formula, only in windows where a small number of bacterial cells were found, namely for suspensions with an approximate concentration of 10^3-10^4 cells/ml. Further, these suspensions were pipetted, on a slide with eight windows and worked by indirect immunofluorescence, to confirm whether this first determination of the bacterial concentration was correct. The cells in 40 fields of each window were counted, then the values in the 8 windows of the slide were summed up. The resulting number was divided by the number of fields read over the whole slide, resulting in the average number of cells per slide. By applying the above formula, the bacterial concentration of the used suspensions can be determined. The data obtained after reading under the epifluorescence microscope and bacterial concentration are listed in Table 2.

Consequently, the suspensions where the very large number of bacteria did not allow the determination of the bacterial concentration shall be regarded as suspensions with a concentration higher than 10^3 cells/ml, and the suspensions where no bacterium was detected, have been considered suspensions with a concentration less than 10^3 cells/ml. Bearing in mind that decimal dilutions were made, it was possible to determine the bacterial concentration in the dilutions where cell counting could not be carried out due to the excessive number of cells per window. The bacterial concentrations used in the present experiment are shown in Table 3.

Table 1

Determination of the average number of cells per window and the bacterial concentration in suspensions of *Clavibacter michiganensis* subsp. *michiganensis*

Strain	Number of cells / window										Medium number of cells (c) / window Bacterial concentration (N) / ml
	g^1	g^2	g^3	g^4	g^5	g^6	g^7	g^8	g^9	g^{10}	
***Cmm* PD 223**	+	+	+	+	27	5	-	-	-	-	g^6 → c = 0,12 N= 6973,14 = 6,97·10^3
***Cmm* NCPPB 2979**	+	+	+	+	+	61	7	-	-	-	g^7 → c = 0,17 N= 9878,61 = 9,87·10^3
***Cmm* 19-3857**	+	+	+	+	57	7	-	-	-	-	g^6 → c = 0,17 N= 9878,61= 9,87·10^3
***Cmm* 19-3858**	+	+	+	+	+	+	43	7	-	-	g^8 → c = 0,17 N = 9878,61= 9,87·10^3
***Cmm* 19-3861**	+	+	+	+	+	35	6	-	-	-	g^7 → c = 0,15 N= 8716,42 = 8,71·10^3
***Cmm* 19-4088**	+	+	+	29	7	-	-	-	-	-	g^5 → c = 0,17 N= 9878,61 = 9,87·10^3
***Cmm* 19-4326**	+	+	+	+	+	41	4	-	-	-	g^7 → c = 0,10 N= 5810,95 =5,81·10^3
***Cmm* 19-4327**	+	+	+	+	52	6	-	-	-	-	g^6 → c = 0,15 N= 8716,42 = 8,71·10^3
C – (sterile water)	-	-	-	-	-	-	-	-	-	-	c = 0 N= 0

Cmm - Clavibacter michiganensis subsp. *michiganensis*; "C – " negative control
"– " negative; "+" positive (the number of cells is too large and cannot be read);
"g^1" - window with parent suspension; "$g^{2\text{-}10}$" - windows with decimal dilutions;
c = number of cells in a window ÷ no. of fields per slide (40 fields); **N** = c · 58109,5.

Table 2

Determination of the average number of cells per slide and the bacterial concentration in suspensions of *Clavibacter michiganensis* subsp. *michiganensis*

Strain	Number of cells / window								Medium number of cells (c) / slide Bacterial concentration (N) / ml
	g^1	g^2	g^3	g^4	g^5	g^6	g^7	g^8	
***Cmm* PD 223**	7	9	5	0	11	2	2	3	c = 0,12 N = 6973,14 = 6,97 · 10^3
***Cmm* NCPPB 2979**	10	3	8	7	1	3	3	7	c = 0,13 N = 7554,23 = 7,54 · 10^3
***Cmm* 19-3857**	12	6	8	5	7	7	6	4	c = 0,17 N = 9878,61 = 9,87 · 10^3
***Cmm* 19-3858**	7	8	5	7	9	2	6	5	c = 0,15 N = 8716,42 = 8,71 · 10^3
***Cmm* 19-3861**	2	0	1	9	1	3	10	8	c = 0,10 N = 5810,95 = 5,81 · 10^3
***Cmm* 19-4088**	4	3	7	10	3	0	15	14	c = 0,17 N = 9878,61 = 9,87 · 10^3
***Cmm* 19-4326**	3	7	4	3	7	6	4	7	c = 0,12 N = 6973,14 = 6,97 · 10^3
***Cmm* 19-4327**	10	7	4	9	3	6	7	6	c = 0,16 N = 9297,52 = 9,29 · 10^3
C – (sterile water)	0	0	0	0	0	0	0	0	c = 0 N = 0

Cmm - Clavibacter michiganensis subsp. *michiganensis*; "C – " negative control ;
"$g^{1\text{-}8}$" windows with *Cmm* bacterial suspensions, having the approximate concentration of 10^3 cells/ml;
no. of fields per slide = 40 fields/ window · 8 windows = 320 fields per slide;
c = (g^1+g^2+g^3+g^4+g^5+g^6+g^7+g^8) ÷ no. of fields per slide (320 fields); **N** = c · 58109,5

Table 3

Establishing of bacterial concentration in suspensions of *Clavibacter michiganensis* subsp. *michiganensis* used

Strain	Bacterial concentration/ml									
	1	**2**	**3**	**4**	**5**	**6**	**7**	**8**	**9**	**10**
***Cmm* PD 223**	**10^8**	10^7	10^6	10^5	10^4	**10^3**	-	-	-	-
***Cmm* NCPPB 2979**	**10^9**	10^8	10^7	10^6	10^5	10^4	**10^3**	-	-	-
***Cmm* 19-3857**	**10^8**	10^7	10^6	10^3	10^4	**10^3**	-	-	-	-
***Cmm* 19-3858**	**10^{10}**	10^9	10^8	10^7	10^6	10^5	10^4	**10^3**	-	-
***Cmm* 19-3861**	**10^9**	10^8	10^7	10^6	10^5	10^4	**10^3**	-	-	-
***Cmm* 19-4088**	**10^7**	10^6	10^3	10^4	**10^3**	-	-	-	-	-
***Cmm* 19-4326**	**10^9**	10^8	10^7	10^6	10^3	10^4	**10^3**	-	-	-
***Cmm* 19-4327**	**10^8**	10^7	10^3	10^5	10^4	**10^3**	-	-	-	-
C – (sterile water)	-	-	-	-	-	-	-	-	-	-

Cmm - Clavibacter michiganensis subsp. *michiganensis*; "C – " negative control;
"1" parent suspension; "2-10" decimal dilutions.

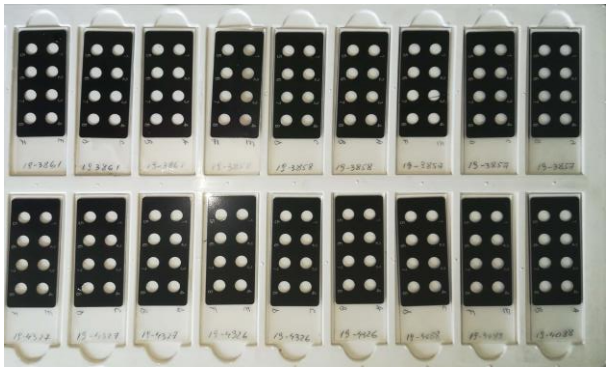

Figure 1. Immunofluorescence slides

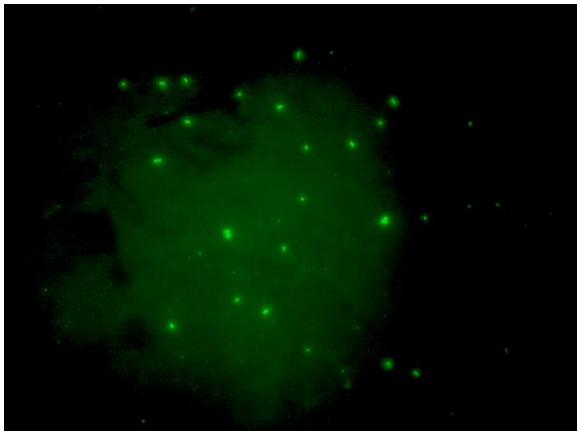

Figure 2. Cells of *Clavibacter michiganensis* subsp. *michiganensis* with living fluorescence

CONCLUSIONS

This reseach demonstrates that *Clavibacter michiganensis* subsp. *michiganensis* could be detected in almost all bacterial dilutions which were used. Thus, after readings under the epifluorescence microscope and calculation performed, it was checked that the minimum limit of detection of the indirect immunofluorescence method is equal to 10^3 cells/ml, as it is mentioned in the EPPO protocol "PM 7/97 (1) Indirect Immunofluorescence test for plant pathogenic bacteria". Determination of a bacterial concentration is important in validation/verification different laboratory tests.

Consequently, the immunofluorescence test is a sensitive assay, being able to detect bacteria even in suspensions with a low bacterial concentration.

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