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PATHOGENICITY AND VIRULENCE OF SOME STRAINS OF *CLAVIBACTER MICHIGANENSIS* SUBSP. *MICHIGANENSIS*, FROM ROMANIA

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Abstract

The harmful organism that produces the "bacterial canker of tomato" (Clavibacter michiganensis subsp. michiganensis) produces a hypersensitivity reaction by infiltration a bacterial suspension in the leaves of Nicotiana tabacum plants and leads to the appearance of characteristic symptoms of the disease by inoculation a bacterial suspension in stems of Solanum lycopersicum seedlings. Analysis of eight isolates of Clavibacter michiganensis subsp. michiganensis, from five counties of the country, in 2019, established the existence of strains with different pathogenicity and virulence.

Key words*: bacteria, hypersensitivity, patogenicity, vascular, strain.*

INTRODUCTION

Clavibacter michiganensis is the only recognized species of the genus *Clavibacter* and it contains nine host-specific species, namely: *Clavibacter michiganensis* subsp. *michiganensis* ("bacterial canker of tomato"), *Clavibacter michiganensis* subsp. *insidiosus* ("bacterial wilt of alfalfa"), *Clavibacter michiganensis* subsp. *sepedonicus* ("ring rot of potato"), *Clavibacter michiganensis* subsp. *nebraskensis* ("wilt of maize"), *Clavibacter michiganensis* subsp. *tessellarius* (infects wheat), *Clavibacter michiganensis* subsp. *phaseoli* (infects bean), *Clavibacter michiganensis* subsp. *capsici* (infects pepper), *Clavibacter michiganensis* subsp. *californiensis* and *Clavibacter michiganensis* subsp. *chilensis* (infects tomato) (Yasuhara-Bell & Alvarez, 2015).

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*) is a monophyletic species, being clearly separated from other pathogenic and non-pathogenic *Clavibacter* species, with which it can share the same habitat (Jacques et al., 2012). It is one of the pathogenic bacteria that causes significant damage to tomato crops. It attacks not only tomato plants but also others *Solanaceae* plants. Occasionally, it attacks non-solanaceous plants (pepper, corn, peas, beans and onions) (https://gd.eppo.int/taxon/CORBMI/hosts; https://efsa.onlinelibrary.wiley.com/doi/epdf/10.2903/j.efsa.2014.3721; Calle-Bellido et al., 2012). It is an aerobic bacteria, Gram-positive, usually non-motile, curved rod shape and encapsulated (Severin & Iliescu, 2006).

It was the first isolated and describe on the tomato plants, in 1910, by Smith, in North America (Singh & Bharat, 2017; Fatmi et al., 2017; Valenzuela et al., 2018). In Romania, it was reported, in Ilfov county, by Elena Bucur, in 1955 (Marinescu et al., 1986; Rădulescu et al., 1970).

Throughout time artificial inoculations with this bacteria have been carried out at different host or non-host plants, for example: *Avena sativa*, *Citrullus lanatus*, *Cucumis sativus*, *Helianthus annuum*, *Hordeum vulgare*, *Secale cereale*, *Triticum aestivum*, *Cyphomandra betacea*, *Lycopersicum pimpinelifolium* (*Solanum racemigerum*), *Nicotiana glutinosa*, *Solanum humboldii*, *Solanum muricatum*, *Solanum nigrum* var. *guineese*, *Solanum pruniforme*, *Solanum tuberosum*, *Solanum racemiflorum*, *Solanum melongena*, *Capsicum annuum* and *Phaseolus vulgaris* (https://gd.eppo.int/taxon/CORBMI/hosts; Severin & Iliescu, 2006).

Behavior and resistance of tomato plants to the attack of *Clavibacter michiganensis* subsp. *michiganensis* were highlighted by such

artificial inoculations, obtaining different results: irregular results after root inoculation and uniform results after inoculation of bacterial suspensions in the stems of fourteen-year-old plants. For differentiating sensitive plants from resistant plants, the most suitable method is the method of cutting the petiole, which gives a partial resistance, due to bacterial inhibition. And in order to determine the optimal period of attack with this bacteria, the method of inoculation by splashing or injury in the cotyledon stage is the most recommended (Severin & Iliescu, 2006).

Temperature required for development of *Clavibacter michiganensis* subsp. *michiganensis* is between 20 and 30^0C, with an optimal growth at 25^0C and a maximum survival at 50^0C. The pH also influences the growth and development of this bacteria. A pH of 7-8 is more favorable for the development of the disease than a pH of 5 (Sen et al., 2015).

The pedoclimatic conditions of the environment influence the manifestation of the symptoms of the disease, obtaining:

- a vascular attack at high temperatures (more than 23^0C) and low rainfall;
- a fruit attack at low temperatures (less than 21^0C) and heavy rainfall;
- a stronger attack if the soil temperature is higher (above the optimum growth temperature) and a slower attack if the soil temperature is lower (below the optimal growth temperature);
- an external infection of plants at medium temperatures and high humidity (Rădulescu et al., 1970).

To determine whether a strain is pathogenic or not in plants, **hypersensitivity reaction** can be performed on certain sensitive plants. The hypersensitivity reaction occurs when the tissues of a sensitive plant are invaded by some phytopathogenic agents, which cause necrosis on the inoculated leaves. If a young bacterial suspension of *Clavibacter michiganensis* subsp. *michiganensis* infiltrates into the intracellular spaces of the leaves of ***Mirabilis jalapa*** or ***Nicotiana tabacum***, under certain temperature conditions (18-43^0C), an inoculated plant response (collapse or internervurian necrosis) is obtained, after 24 hours on the leaves of *Nicotiana tabacum* and after 48-72 hours on the leaves of *Mirabilis jalapa* (Gitaitis, 1990; Montesinos, 2000; Burokiené et al., 2005; Shaker, 2014).

Studies show that for a better understanding of the mechanism of disease production and evolution, nineteen bioluminescent strains of *Clavibacter michiganensis* subsp. *michiganensis* were isolated. All mutant strains induced hypersensitivity reaction in *Mirabilis jalapa* and caused wilting symptoms in tomato plants (Xu et al., 2010).

For establishing the virulence of the different strains of *Clavibacter michiganensis* subsp. *michiganensis* **pathogenicity tests** can be performed. The pathogenicity test is performed in the greenhouse and allows the artificial reproduction of the disease, under certain conditions controlled by temperature and humidity. The EPPO protocol "PM 7/42 (3) *Clavibacter michiganensis* subsp. *michiganensis*", recommends two methods of inoculation: **the test in tomato plantlets** and **the cotyledon test**. The second method is performed, in particular, when the tomato isolates did not produce wilting through the seedling test. The presence of symptoms of wilting of the infected plants or the appearance of wounds on the stems (in the case of the seedling test) and the appearance of white blisters or craters on the surface of the cotyledons (in the case of cotyledon test) indicate the presence of infection with *Clavibacter michiganensis* subsp. *michiganensis*. The absence of any symptoms suggests a negative reaction, which means that the disease has not reproduced and the inoculated suspensions are not contaminated with *Clavibacter michiganensis* subsp. *michiganensis*.

Pathogenicity tests performed on tomato seedlings allowed different strains of *Clavibacter michiganensis* subsp. *michiganensis* to be grouped into strains with high, medium and low virulence (Burokiené et al., 2005).

In this case we aim to analyze and establish the pathogenicity and virulence of some strains of *Clavibacter michiganensis* subsp. *michiganensis*, in Romania, by the hypersensitivity test and the pathogenicity test.

MATERIALS AND METHODS

For this experiment were used two reference strains of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm* PD 223; *Cmm* NCPPB 2979) and eight isolates of *Clavibacter michiganensis* subsp. *michiganensis,* from *Lycopersicon lycopersicum*, from five county of Romania: Argeș (cv. Colibri - *Cmm* 19-3857 and *Cmm* 19-3861; cv. Paris - *Cmm* 19-3858), Dolj (cv. Prekos - *Cmm* 19-4088), Suceava (cv. Colibri - *Cmm* 19-4326; cv. Kyveli - *Cmm* 19-4327), Hunedoara (cv. Kingset - *Cmm* 19-4735), Satu-Marte (cv. "Inimă de bou" - *Cmm* 19-4819).

The contaminated plants were taken by the phytosanitary inspectors from the Phytosanitary Offices and sent to the National Phytosanitary Laboratory, within the National Phytosanitary Authority, Romania. After conducting laboratory analyses, they were declared contaminated with *Clavibacter michiganensis* subsp. *michiganensis*.

The strains used were kept in the freezer at -80^{0}C, on PROTECT^{TM} beads. To obtain bacterial colonies, from each strain we used two beads contaminated with *Clavibacter michiganensis* subsp. *michiganensis*. They were placed on the surface of a common culture medium YPGA (Difco yeast extract 5 g, Difco Bacto peptone 5 g, D(+) glucose 10 g, Difco Bacto agar 15 g). Petri dishes inoculated with the strains of interest were incubated at 27^{0}C, for 5 days. Colonies with typical morphology were collected and used to make of bacterial suspensions in one ml sterile water. The suspensions had slight turbidity and were first tested by indirect immunofluorescence to determine bacterial concentration. Bacterial suspensions with an approximate concentration of 10^7-10^8 ufc/ml were used as such, while suspensions with a concentration greater than 10^8 ufc/ml were diluted to the desired concentration. The bacterial suspensions thus obtained were used to induce the hypersensitivity reaction and to performe the pathogenicity test.

For the hypersensitivity reaction, the bacterial suspensions were infiltrated, with a hypodermic syringe, in intercellular spaces of completely developed leaf by *Nicotiana tabacum* cv. White Barley. Plants are inoculated and incubated at 27-30^{0}C and followed for 1-4 days, until symptoms appear.

For the pathogenicity test, there were used ten plants of *Solanum lycopersicum* cv. Moneymaker and nine plants of *Solanum melongena* cv. Black Beauty. The plants used for inoculation were young plants in the stage of two-three true leaves. These were inoculated with a bacterial suspension of *Clavibacter michiganensis* subsp. *michiganensis*, in five points of the stems, by injection. The plants thus inoculated were incubated in the quarantine greenhouse, at a temperature of 26-28^{0}C, humidity 60-72%, alternation day/night of 15/9 hours and they were observed for 25 days.

After 25 days, the stems of the eggplants and tomato plants inoculated were cut, with a scalpel, at the soil surface. The leaves were removed and the remaining material was cut and then milxed using a Homex. To these material, a sufficient amount of sterile water was added and then stirred for 20-30 min. The extract obtained was tested by indirect immunofluorescence, according to the EPPO protocol "PM 7/98 Indirect Immunofluorescence test for plant pathogenic bacteria" and were observed under the fluorescence microscope.

RESULTS AND DISCUSSIONS

Hypersensitivity reaction. The leaves of the plants of *Nicotiana tabacum* cv. White Barley inoculates were evaluated after 24, 48, 72 and 96 hours. In the areas where the bacterial suspension was infiltrated, different reactions were obtained, such as: yellowing, necrosis and collapse (Table 1).

By observing the reaction of the inoculated tobacco leaves it was found:

- the strains of *Cmm* PD 223, *Cmm* 19-3857 and *Cmm* 19-4326 are highly pathogenic strains, because an internervurian collapse was obtained after 24 hours; after 96 hours, the internervurian collapse was intensified and browned, with or without the yellowing of the areas around it;
- the strains of *Cmm* NCPPB 2979, *Cmm* 19-3861, *Cmm* 19-4735 and *Cmm* 19-4819 are medium pathogenic strains because initially, after 24 hours, a slight collapse or necrosis of

the inoculated areas occurred, followed by an intensification of the necrosis areas in the following hours and by the yellowing of the areas adjacent to them;

Table 1. Hypersensitivity reaction - the evolution of symptoms

Strains of *Cmm*	Leaves of *Nicotiana tabacum* cv. White Barley			
	24 hours	48 hours	72 hours	96 hours
Positive control *Cmm* NCPPB2979				
Positive control *Cmm* PD223				
Cmm 19-3857				
Cmm 19-3858				
Cmm 19-3861				
Cmm 19-4088				
Cmm 19-4326				
Cmm 19-4327				
Cmm 19-4735				
Cmm 19-4819				

- the strain of *Cmm* 19-3858 is a low pathogenic strain whereas, 24 hours, a necrosis of the inoculation points appeared, followed after 72 hours by the yellowing of the inoculated areas;
- the strains of *Cmm* 19-4088 and *Cmm* 19-4327 are very low pathogenic strains as they did not produce any reaction within 24 hours; the yellowing of the inoculated areas starting after 48 hours;
- the negative control did not show any reaction.

Pathogenicity test. Bacterial suspensions of *Clavibacter michiganensis* subsp. *michiganensis* used produced in the seedlings of *Solanum lycopersicum* cv. Moneymaker in which they were inoculated, characteristic symptoms of "Bacterial canker of tomato".
At 7 days after inoculation, in some plants, the margins of the leaflets turned upwards, the entire leaflet bent down, along the median rib, the petioles of the affected leaves curved downwards, leading to the bending of the whole leaves. The stems of plants were deformed and the plants with symptoms remained smaller compared to those where the symptoms have not manifested yet (Figure 1).

Figure 1. Foliar symptoms of *Clavibacter mchiganensis* subsp. *michiganensis* on *Solanum lycopersicum*

After 10-15 days, initially, at the inoculation points of the stems appeared small, lenticular cankers, which extended along the stem causing longitudinal cracks, in advaced stages (Figure 2).

Figure 2. Symptoms of *Clavibacter mchiganensis* subsp. *michiganensis* on stem plants of *Solanum lycopersicum*

After 20-25 days, some plants died and at others plants the leaves were completely wilted and they remained attached to the plants.
Therefore, at 7 days after inoculation, the procent of plants with symptoms was:

- 90% affected plants - at plants infected with strains of *Cmm* PD 223, *Cmm* 19-3857 and *Cmm* 19-4326;
- 70% affected plants - at plants contaminated with strains of *Cmm* NCPPB 2979, *Cmm* 19-3861 and *Cmm* 4819;
- 30% affected plants - at plants inoculated with the strains of *Cmm* 19-3858 and *Cmm* 19-4327;
- 10% affected plants - at plants infected with the strains of *Cmm* 19-4088 and *Cmm* 19-4735;
- no symptoms plants at negative control.

We considered that a strain is more virulent than other if the number of plants showing symptoms is higher and the number of days after inoculation until appearance of symptoms is smaller. And we considered that a strain is less virulent if the symptoms appear in a longer period of time than those on other plants.

Therefore, the studied strains can be grouped as follows:

- with high virulence - *Cmm* PD 223, *Cmm* 19-3857 and *Cmm* 19-4326;
- with medium virulence - *Cmm* NCPPB 2979, *Cmm* 19-3861 and *Cmm* 19-4819;
- with low virulence - *Cmm* 19-3858 and *Cmm* 19-4327;
- with very low virulence - *Cmm* 19-4088 and *Cmm* 19-4735.

In the case of artificial infection with *Clavibacter michiganensis* subsp. *michiganensis* of the seedlings of *Solanum melongena* cv. Black Beauty, the foliar symptoms were almost completely absent. Only a few leaves showed symptoms of wilting and interveinal chlorosis (Figure 3).

Figure 3. Foliar symptoms of *Clavibacter mchiganensis* subsp. *michiganensis* on *Solanum melongena*

Also, the simptom of deformation of the stem of the inoculated eggplant was almost absent. Only one or two inoculated plants showed such a symptom. In contrast, all plants showed lenticular lesions on the stems at the place of inoculation, lesions that expanded and led to the appearance of longitudinal cracks (Figure 4).

Figure 4. Symptoms of *Clavibacter mchiganensis* subsp. *michiganensis* on stem plants of *Solanum melongena*

Regarding the analysis of tomato and eggplant extracts obtained after the grinding of stems of these inoculated plants and after performing the immunofluorescence test, the presence of the target bacteria was found in all the analysed extracts and their absence in the negative control. It was obvious that the number of bacteria in tomato extracts was much higher than in eggplant extracts. Therefore, the bacterial concentration was much higher in *Solanum lycopersicum* plants, compared to those of *Solanum melongena*.

CONCLUSIONS

Through the **hypersensitivity reaction** it was established that all the strains analyzed were pathogenic. The reactions on the leaves of *Nicotiana tabacum* cv. White Barley were variable, as follows: the pathogenic strains caused a faster reaction whereas the less pathogenic strains caused a later reaction. The stronger pathogenic strains caused, after 24 hours, collapse and necrosis in the areas where the bacterial suspension was infiltrated, and the low pathogenic strains caused, after 48-72 hours, only a yellowing of the inoculated areas.

Pathogenicity test allowed the establishment the virulence of the studied strain, depending on the speed of the onset of symptoms. Any studied strain was not devoid of virulence. Low virulent strains of *Clavibacter michiganensis* subsp. *michiganensis* caused a slower reaction and a delay in the onset of symptoms on test plants of *Solanum lycopersicum*, while more virulent strains caused a faster onset of symptoms. As established Burokiené'study, in 2005, the strains of *Clavibacter michiganensis* subsp. *michiganensis* has different degrees of

virulence. We can conclude that, the strains analyzed also showed different degree of virulence, namely: high, medium, low and very low virulence.

Solanum melongena plants showed very low foliar symptoms, lacking in intensity and aggressivity. They appeared later compared with the foliar symptoms of *Solanum lycopersicum* plants. The wounds on the stems are similar to the two types of test plants used for pathogenicity. We can say that the intensity of the symptoms on the stem was much lower in *Solanum melongena* plants, compared to those on *Solanum lycopersicum* plants. The indirect immunofluorescence analysis revealed the existence of a larg number of bacteria in tomato plants compared to eggplant.

Regarding the reproduction of the disease on test plants, the EPPO protocol "PM 7/42 (3)" recommends the use of tomato plants. However, we can conclude that although the eggplants have an apparent resistance to the attack of the bacterium *Clavibacter michiganensis* subsp. *michiganensis*, they can be used as test plants when tomato test plants are not available.

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