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PATHOGENIC CAPACITY OF CLAVIBACTER MICHIGANENSIS SUBSP. MICHIGANENSIS ISOLATED FROM TOMATO ON DIFFERENT SOLANACEAE: TOMATO (*SOLANUM LYCOPERSICUM*), POTATO (*SOLANUM TUBEROSUM*), EGGPLANT (*SOLANUM MELONGENA*), PEPPER (*CAPSICUM ANNUUM*), TOBACCO (*NICOTIANA TABACUM*) AND PETUNIA (*PETUNIA HYBRIDA*)

Marquez-Zequera
`` Centro de Investigación sobre Alimentación y Desarrollo``
``Universidad Autonoma de Sinaloa``

Cruz-Lachica I
`` Centro de Investigación sobre Alimentación y Desarrollo``

Enríquez-Verdugo
``Universidad Autonoma de Sinaloa``

Torres-Valdez J B
`` Centro de Investigación sobre Alimentación y Desarrollo``

Gaxiola-Camacho S M
``Universidad Autonoma de Sinaloa``

Osuna-Garcia L A
`` Centro de Investigación sobre Alimentación y Desarrollo``

Garzon-Tiznado J A
``Universidad Autonoma de Sinaloa``

Gomez-Gonzalez G
`` Centro de Investigación sobre Alimentación y Desarrollo``

Garcia-Estrada R S
`` Centro de Investigación sobre Alimentación y Desarrollo``

Abstract: Clavibacter michiganensis subsp. michiganensis (*Cmm*), is a plant pathogenic actinomycete that causes wilting and bacterial cancer of tomato (Solanum lycopersicum L.). The Clavibacter genus includes bacteria of economic importance, causing great losses because they infect crops such as corn, alfalfa, potatoes, peppers, tomatoes and wheat and are quarantined worldwide. The objective of this research was to determine the pathogenic capacity of Cmm in different Solanaceae species of economic importance in Mexico. For this reason, inoculations with Cmm were carried out on tomato (Solanum lycopersicum), petunias (Petunia hybrida), potato (Solanum tuberosum), eggplant (Solanum melongena), pepper (Capsicum annuum) and tobacco (Nicotiana tabacum) plants. The TM 68 strain conserved in the strain collection of the Phytopathology laboratory of CIAD, A.C. was used. Culiacán, which was isolated from tomato plants from Ciudad Obregón Sonora, Mexico, and characterized morphologically and molecularly. The results indicate that, in inoculated tomato plants, the presence of symptoms was observed 13 days after inoculation (dai), showing characteristic symptoms such as yellow and rolled leaves, which at 24 dai were dry; In addition, general wilting is observed until finally the plant dies. Regarding the other solanaceous plants, the petunia plants presented symptoms of yellowing at the tips of the leaves and curling at 67 dai. On the other hand, the potato plants showed yellowing at 77 days and the tubers showed a brown ring; and the pepper plants showed strong defoliation. Finally, the eggplant and tobacco plants did not show any symptoms. At 60 days after specific detection of the bacteria using the PCR technique, it was determined positive in the six Solanaceae species. Therefore, it is concluded that the bacterium Clavibacter michiganensis subsp. michiganensis has the ability to reproduce in

other solanaceous plants other than tomatoes, although it may or may not cause disease symptoms.

Keywords: *Cmm*, Solanaceae, Bacterial cancer, Pathogenicity

INTRODUCTION

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*), It is a gram-positive aerobic bacterium and is the causal agent of bacterial cancer in tomato(*Solanum lycopersicum* Mill.). This disease was first described in 1910 in Michigan, USA (Eichenlaub et al., 2006). As a result of the severity of the disease and due to economic losses due to the decrease in production, Cmm is considered a quarantined organism by the European Union and many other countries (De León et al., 2008). Cmm infection begins through wounds and natural openings such as stomata, causing a decrease in the yield of tomato production worldwide (Carlton et al., 1998).

Secondary infection can take place once the crop is established and occurs mainly due to cultural practices such as pruning, tutoring, contact between diseased and healthy plants, splashes during pesticide applications, irrigation, etc. (Ricker and Riedel, 1993). The symptom of wilting on the leaves is the most commonly observed; The damage begins in the oldest leaves, where yellowing of the margins towards the leaf blade appears, the affected tissues gradually die and the leaf blade dries out. The disease progresses from the lower part upwards and the foliage becomes burned or blackened. Darkening can develop on the petioles of the leaves as well as on the stems (Vega and Romero, 2016). When cutting the stems longitudinally, the vascular tissues can be seen to acquire a moist, yellow or orange appearance, and in some cases even a dark color. It is worth mentioning that, when the bacteria are found in high concentrations in these tissues, the marrow can be easily separated (Jahr et al., 2000). In infected fruits, a spot known as "bird's eye" is occasionally observed, which appears as a small dark spot, surrounded by a white halo (Chang et al., 1992; Werner et al., 2002). Another characteristic symptom in fruits is the presence of yellow and/or orange dots in the area of the vascular tissues of the peduncle when the fruit is separated from diseased plants by Cmm. In this case, the points (xylem and phloem tissues) have a wet appearance and are deep yellow and in some cases orange. In this regard, great variability has been observed in the symptoms caused by Cmm in solanaceous plants and this depends mainly on the susceptibility of the host cultivar, the virulence of the strain and the environmental conditions that favor or do not favor the development of the disease. (Smirnov et al., 2023). The bacteria moves through the vascular tissues of the fruit, reaches and enters the internal part of the seeds, these being the main source of dissemination over long distances where it is transmitted to the plant that gives rise and from there to the crop in general. In the final stage of the disease, the entire plant wilts and dies (Sen et al., 2015).

The genus Clavibacter has been grouped into five subspecies which cause diseases in different crops of economic importance: in tomato (C. michiganensis subsp. michiganensis, Cmm), in corn (C. michiganensis subsp. nebraskensis, Cmn), in potato (C. michiganensis subsp. sepedonicus, Cms) in alfafa (C. michiganensis subsp, insidiosus, Cmi) and in peppers (C. michiganensis subsp. capsici, Cmc) (Tambong, 2017).

According to various research works, it has been reported that Cmm has the capacity to cause damage to other crops in addition to its main host, which is tomato. In this sense, phenotypic variants of Cmm were isolated from pepper fields and pepper seeds during quarantine inspections in the United States, Europe, and other countries. All strains isolated from this crop produced orange colonies with a less mucoid consistency than typical Cmm strains isolated from tomato; however, the results of ELISA, fatty acid analysis, 16S rDNA sequencing, and PCR analysis showed that all pepper isolates were similar enough to be identified as Cmm. Likewise, inoculation tests of the tomato and pepper isolates showed that the former caused severe wilting and canker in the tomato, but they only caused canker and did not wilt the pepper and bell pepper; however, pepper isolates did not cause wilting, even in tomato. In that sense, Yim et al. (2012) suggest that pepper isolates may represent a separate Cmm population that has evolved within the confines of this host.

In 5 regions of Russia during the years 2011 to 2017 Ignatov et al. (2019) isolated Clavibacter from yellow pepper plants and diseased potato tubers. Potato plants exhibited yellowing, leaf necrosis, wilting of leaves and whole plants, and brown veins around the eyes of the tuber were observed in cross sections. Isolates from each diseased potato plant (35 isolates) were identified as Clavibacter michiganensis subsp. michiganensis (Cmm). In this study, the taxon-specific polymerase chain reaction (PCR) technique was used with primers CMM5/CMM6. Sequencing of the 16S rRNA gene (GenBank accession numbers MH035728.1 to MH035762.1) showed a >99% relationship to the Cmm type strain NCPPB2979T. This was the first report of Cmm affecting potatoes in this country, so there is a need to know the ability of the bacteria to infect other species of solanaceous plants, which will improve the understanding of its pathogenesis.

MATERIALS AND METHODS

CMM STRAIN USED FOR PATHOGENICITY TESTING

The Cmm strain isolated from tomato from Mexico identified as TM 68, which showed a high degree of pathogenicity in this crop and which was previously characterized morphologically and molecularly (GenBank accession number: MK816895), was reactivated in Mueller Hinton artificial medium.

SEEDLING PRODUCTION

The seeds of the six nightshades were sown: tomato (Solanum lycopersicum), petunias (Petunia hybrida), potato (Solanum tuberosum), eggplant (Solanum melongena), pepper (Capsicum annuum) and tobacco (Nicotiana tabacum), for which soil previously sterilized at 121°C for one hour.

INOCULATION OF SEEDLINGS AND VISUALIZATION OF DISEASE SYMPTOMS

Two pots containing 5 seedlings each were used for each solanaceous species evaluated. Uninoculated control seedlings were included in the analysis. For inoculation, a sterile wooden stick was used, the tip of which was impregnated with bacterial cells of the Cmm strain and placed in the axil of the seedlings (Figure 1). The pots were maintained under greenhouse conditions with the agronomic management of irrigation and fertilization required for the different species. Symptom assessment was performed daily after inoculation.

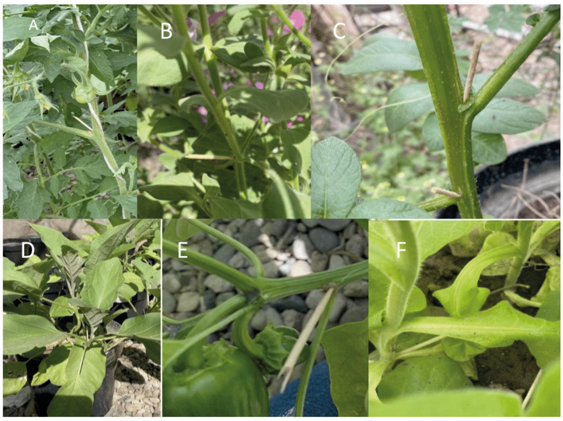

Figure 1. Inoculated seedlings of the different nightshades A): tomato, B): petunia, C): potato, D): eggplant, E): pepper and F): tobacco.

RAPID DETECTION OF CMM BACTERIA WITH THE USE OF IMMUNOSTRIPS AND REISOLATION IN MUELLER HINTON CULTURE MEDIUM

A tissue sample was taken from each of the 6 solanaceous crops and the test was performed with the Cmm-specific immunostrips from the Agdia brand, according to the instructions suggested by the manufacturer. To verify that the bacteria was viable, it was reisolated from the immunostrip buffer from which a sample was taken and sown in Mueller Hinton culture medium.

DETECTION OF *CMM* BY PCR IN INOCULATED PLANTS

In order to confirm the presence/absence of Cmm and verify the detection sensitivity of the immunostrips, an endpoint PCR was run in all evaluations. To this end, DNA extraction was carried out from plants inoculated with the TM 68 strain of the six Solanaceae species and from the control plants using the method using CTAB (Cetyltrimethylammonium Bromide), following the protocol described by Voigt et al. (1999).

Subsequently, specific detection was carried out by PCR with the primers Cm3 (5'-CCTCGTGAGTGCCGGGAACGTAT-3') y Cm4 (5'-CCACGGTGGTTGATGCTCGCGA-3') under the amplification conditions proposed by Sousa et al. (1997), with modification of the annealing temperature and time in the three phases of PCR (denaturation, annealing and elongation) as described below: initial denaturation of 94°C for 5 min, followed by 40 cycles of 94°C for 1 min, 62°C for 1 min, 72°C for 30 s and a final extension of 72°C for 5 min. The PCR was carried out in a Bio-Rad model T-100 thermocycler.

Finally, the PCR products were visualized on 1% agarose gel in an electrophoresis chamber (BioRad) with the conditions of 80 V, 400 mA for 80 min.

RESULTS AND DISCUSSIONS

The first symptoms recorded were observed in the inoculated tomato plants at 13 dai where the presence of yellow and rolled leaves was manifested. Subsequently, at 24 dai the plants showed general wilting and the foliage showed blighting which was more intense in the older leaves and increased as the days went by. The detection of the bacteria was corroborated with the use of immunostrips, which gave a positive result (Figure 2A) and from the buffer, seedings were carried out in Mueller Hinton culture medium and the bacteria were correctly re-isolated (Figure 3A). Finally, when making longitudinal cuts in the stems, the vascular tissues were observed to be moist and yellow in color, where the stem cover was easily detached between nodes. The symptoms observed in tomato plants corresponded to those reported by various authors such as Jahr et al. (2020), Vega and Romero (2016), Chalupowicz et al. (2012) and Sen et al. (2015). With respect to the symptoms associated with bacterial cancer disease, it has been reported that the Cmm bacteria require the active secretion of serine proteases from the early stages of the infection and that they facilitate its movement to make bacterial aggregates in the xylem of the plant. tomato, and this way obstructs the vascular bundles, preventing free access of essential nutrients for the normal development of plants (De león et al., 2011).

In pepper plants inoculated with the Cmm bacteria, the characteristic symptoms that commonly occur in tomatoes were not observed; However, at 24 dai, a positive result was obtained in the rapid tests with immunostrips (Figure 2E) from which the strain inoculated in Mueller Hinton culture medium was reisolated (Figure 3B). At 67 dai the plants showed severe defoliation, which differs from the results reported by Yim et al. (2012) where they observed in pepper plants inoculated with a Cmm strain isolated from tomato a minimal development of canker on the stems and leaf blight without wilting; However, it is worth mentioning that its evaluation period was limited to 25 days and that the presence of symptoms is associated with the susceptibility of the host cultivar, the virulence of the strain and the environmental conditions that favor or do not favor the development of the disease. (Smirnov et al., 2023).

For the other inoculated solanaceae, rapid detection was performed with immunostrips at 24 dai; However, these gave negative results (Figures 1B, 1C, 1D and 1F) corresponding to petunia, potato, eggplant and tobacco plants, respectively.

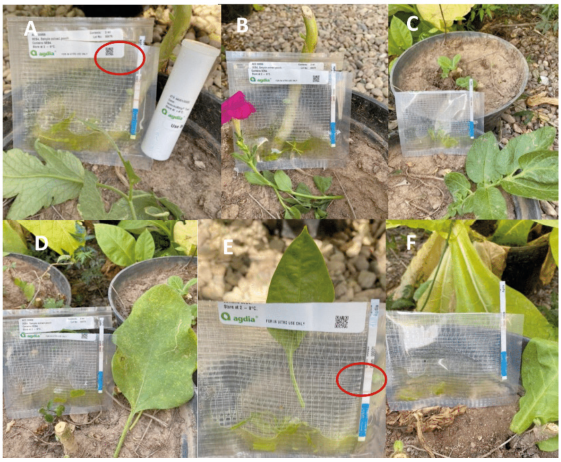

Figure 2. Rapid immunological test for Cmm at 24 dai A): Positive tomato plant (indicated by two intense red bands including the positive control). B): negative petunia plant. C): Negative potato plant. D): Negative eggplant plant. E): Positive pepper plant and F): Negative tobacco plant.

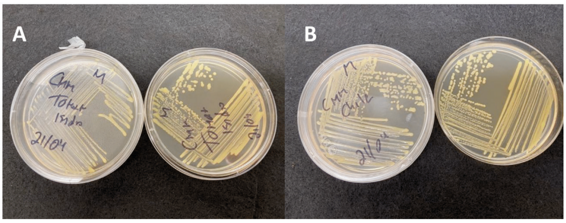

Figure 3. Cmm seeding of immunostrip buffer. A) isolated from tomato plants with symptoms and B) isolated from pepper plant without symptoms of bacterial cancer.

At 60 dai, samples were taken from the 6 different solanaceae inoculated with the TM 68 strain and DNA extraction was carried out to perform a PCR with the specific primers for Cmm (Cm3 and Cm4) that generate a 640 bp amplicon. As a result, positive detection was observed for all inoculated solanaceous plant species. The above confirms the positive result previously obtained for tomato and chili plants and suggests that petunia, potato, eggplant and tobacco plants face Cmm infection showing a certain degree of tolerance, a phenomenon defined by Pagán and García-Arenal (2018) as the host's ability to cope with infections caused by pathogens where the plant moderates the level of their replication and reduces the damage caused by its infection.

Subsequently, seven days after the positive confirmation of the bacteria by PCR, yellowing symptoms were observed on the tips of the leaves towards the stem and flowers in the inoculated petunia plants. In addition, detection by immunological strips and seeding in Mueller Hinton culture medium were also carried out (Figure 4). The above allows us to indicate that petunia plants have an intermediate degree of tolerance to *Cmm* infection, but they do show characteristic symptoms, although in a much longer time than that observed for tomatoes and peppers, the former being their main host and the second with the least affectation, a host already reported (Burokiene et al., 2005 ; Latin et al., 1995 ; Lewis-Ivey y Miller, 2000).

Figure 4. Detection of Cmm in petunia plant. A) Plant with symptoms caused by the bacteria, B) Positive detection with the use of immunostrips and C) Growth of the bacteria in culture medium.

At 77 dai, the final evaluation of the potato, eggplant and tobacco plants was carried out, where the potato plants showed yellowing and dry leaves; as well as, a brown ring when making longitudinal cuts in the tubers (Figures 5A and 5B), which agrees with what was reported by Ignatov et al. (2019) for the symptoms developed by Cmm infection in this crop. Subsequently, a rapid immunological test was performed and this gave a positive result. Furthermore, from the immunostrip buffer, the seeding was carried out in Mueller Hinton culture medium (Figures 5C and 5D). The above confirms the positive result obtained by the PCR technique and also corresponds to the degree of intermediate tolerance described above for petunia plants.

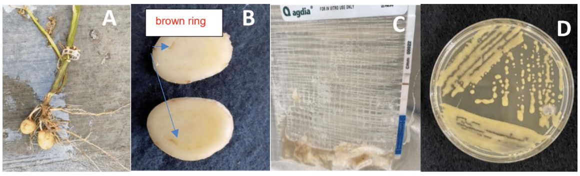

Figure 5. A) Potato plant with fruits and necrotic stem base, B) Potato fruit that has brown rings in the center, C) Rapid test for the detection of the bacteria with a positive result and D) Growth of the bacteria in culture medium.

At the end of the evaluation period at 77 dai, the eggplant and tobacco plants inoculated with the *Cmm* bacteria showed no differences with the non-inoculated plants (negative controls) and although both species gave positive results for the detection of the bacteria by PCR, they did not showed symptoms and the strain was not recovered in culture medium as in the other four solanaceae. The above may indicate that these two species have developed a high degree of tolerance to the *Cmm* infection process in such a way that the bacterial replication thresholds are conserved at such low levels that they are not manifested visually since they are not capable of causing damage.

CONCLUSIONS

In the present work it was determined that the bacteria *Clavibacter michiganensis* subsp. *michiganensis* It is capable of causing disease in other solanaceous plants such as pepper, petunias and potatoes; in addition to tomato plants, where the symptoms develop in a shorter period of time and with greater severity. Although the common symptoms caused by the bacteria did not occur in the pepper plants, defoliation was observed at the end of the experiment. On the other hand, the eggplant and tobacco plants did not show any symptoms; However, they were positive in PCR detection, so this phenomenon may indicate that these species show a very high degree of tolerance to Cmm infection; However, they can be considered hosts and could contribute to the spread of the disease. Of the 6 solanaceae inoculated, only the TM 68 strain was recovered from tomato, pepper, petunia and potato plants, so it could be suggested that in eggplant and tobacco plants the replication of the bacteria is maintained at levels lower than the other solanaceous plants evaluated.

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