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Research Article
Impact of Good Seed and Plant Practices on the Incidence of Bacterial Canker in a Commercial Tomato Nursery

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Abstract

Background and Objective: In tomato commercial nursery, *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of bacterial canker, is mainly transmitted by infected seeds and seedlings, so, microbiological analysis of seedlings to prevent the dissemination of contaminated transplants is crucial in bacterial canker management. The purpose of this study was to evaluate the impact of Good Seed and Plant Practices (GSPP) on the incidence of bacterial canker in grafted tomato seedlings produced in a commercial nursery. **Materials and Methods:** During the 3 years of study (2018, 2019 and 2020), the detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato-seedlings samples was done by dilution plating of stem extract on different culture media. To confirm the molecular identity of the pathogen, one bacterial isolate from each suspected sample was tested by PCR using the primers RZ-ptssk 10 and RZ-ptssk 11. After the PCR analysis, each suspected bacterial isolate was further tested for its pathogenicity by using the cotyledons test on tomato seedlings. **Results:** During the 3 years, more than one thousand tomato seedlings samples were analyzed for *Clavibacter michiganensis* subsp. *michiganensis* contamination and only fifteen samples were identified positive for this bacterial pathogen by using culture isolation on non-selective and semi-selective media. Of these, only four bacterial isolates were positive by using the PCR technique. However, pathogenicity was not confirmed in cotyledon inoculation tests for the four suspected isolates obtained from tomato seedling samples. **Conclusion:** This study demonstrated that the rigorous application of good seed and plant practices standards and implementation of strict sanitary measures significantly reduce the risk of contamination of grafted tomato seedlings by *Clavibacter michiganensis* subsp. *michiganensis* in a commercial tomato nursery.

Key words: Tomato, bacterial canker, *Clavibacter michiganensis* subsp. *michiganensis*, GSPP, Bio-PCR, nursery, bacterial contamination

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Competing Interest: The authors have declared that no competing interest exists.

Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Bacterial canker of tomato (*Lycopersicon esculentum* Mill), is the most common tomato disease caused by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*)[1]. The disease was first reported in 1910 in the United States and has subsequently been observed in most tomato-producing regions worldwide[2-4]. Losses due to this bacterial disease may vary from a few diseased plants to a total loss of production depending on environmental conditions and the agricultural practices used by growers.

Its incidence in Morocco has been reported since 1942[5], where, it is a very important factor in crop yield loss[6,7]. A few commercially grown cultivars have significant tolerance or resistance to *Clavibacter michiganensis* subsp. *michiganensis*[2], while control methods include the planting of disease-free seeds and transplants, removal of infected plants, good hygiene in the nurseries and greenhouses, disinfection of grafting, pruning and pollinating tools and application of chemical pesticides[8,9]. A few infected seedlings in a commercial nursery can initiate severe infection in the field or the greenhouse. Furthermore, the secondary spread of *Clavibacter michiganensis* subsp. *michiganensis* in the commercial nurseries or greenhouses can occur via contaminated equipment used for clipping or grafting practices and workers' hands during the tomato chain production[10]. Thereby, the best way to avoid the disease in the greenhouses is by maintaining the seeds and transplants free of the pathogen[2].

As the control of bacterial canker is very difficult, in many countries commercial tomato nurseries have implemented a safety system, based on Good Seed and Plant Practices[11], to prevent the occurrence of *Clavibacter michiganensis* subsp. *michiganensis* in seed and grafted-tomato seedlings. In Morocco, since 2017, it has been observed a significant increase in the number of commercial tomato nurseries which implemented the GSPP system. Consequently, tomato commercial nurseries which adopt GSPP need to design effective control measures to reduce bacterial contamination risks to an acceptable level and the use of healthy seeds with the GSPP label is an initial guarantee for the success of the production of *Cmm*-free transplants[12].

The purpose of this study was to investigate the impact of implementation of a good seed and plant practices system on bacterial canker occurrence in tomato commercial nursery.

MATERIALS AND METHODS

Site location: The study was conducted in a commercial nursery at the "Domaine Agricole SIRWA", Souss-Massa Region, central west of Morocco, for three consecutive years (2018, 2019 and 2020) and from August, until November, each year. The Souss-Massa Region is the major tomato-growing area of Morocco. The nursery is located at 30°13'38.7"N and 9°31'32.9"W and is above 65 m of sea level. This nursery mainly produces tomato-grafted seedlings and was operating by good seed and plant practices system. The surface of the site GSPP certified nursery is about 6 ha and it is one of the biggest nurseries that specialize in the production of grafted tomato transplants in Morocco (from 5-6 million seedlings per year). In addition, all the tomato seeds (rootstocks and scions) used in the nursery for the production of tomato grafted plantlets were certified by GSPP.

Detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato transplants: The method used in this study for the detection of *Clavibacter michiganensis* subsp. *michiganensis* in symptomless tomato seedlings was elaborated according to the protocols of the International Seed Federation[13] and the European and Mediterranean Plant Protection Organisation[4] which are normally adopted for the detection of *Cmm* on tomato seed. The principles of this method involve the isolation of *Cmm* from tomato seedlings tissue on non-selective and semi-selective media, followed by the identification of presumptive *Cmm*-isolates by PCR and pathogenicity test.

Sampling of tomato plantlets: A microbiological sampling plan was used with the intent of identifying *Cmm* sources in the current GSPP system. The sampling sources were selected based on a literature review related to potential risk factors which may contribute to the *Cmm* contamination of grafted tomato seedlings. These sources were identified as critical sites in the grafted-tomato seedlings production chain (initial infection of scions and rootstocks from contaminated seeds or contamination of seedlings during the grafting and clipping process). The samples were collected from the tomato rootstocks (15 cultivars), scions (15 cultivars) and grafted plantlets (Table 1). Each sample was randomly collected and consisted of 100 stem segments of 2 cm taken from seedlings of the same variety and becoming from the same seed lot[4]. The sampling of grafted or pruned seedlings is delayed until one week after grafting or pruning to allow bacterial proliferation in the tomato tissue of contaminated seedlings[14]. The collected samples were placed in sterile plastic bags, labelled, transported to the Laboratory of Biotechnologies and Plant Protection, Faculty of Sciences, Ibn Zohr University and stored at 4°C before analysis.

Table 1: Varieties and rootstock analyzed in study

Scion "Variety"	Rootstock	Grafted seedling
Angelle	Arazi	Calvi/Tomato
Brentyla	Beaufort	Genio/Protector
Calvi	Embajador	Pristyla/Superpro
Genio	Emperador	Zayda/Kaiser
Pitenza	Gladiator	Angelle/Fusapro
Pristyla	Maxifort	Celia/Estamino
Wafira	Pertiguero	Brentyla/Beaufort
Zayda	Superpro	Pitenza/Embajador
Ateneo	Tomato	Ateneo/Pertiguero
Delyca	Kaiser	Chocostar/Arazi
Vittorino	Estamino	Wafira/Emperador
Adriana	Fusapro	Vittorino/Maxifort
Bellatrix	Rootpremium	Delyca/Gladiator
Celia	Compagnon	Bellatrix/Rootpremium
Chocostar	Protector	Adriana/Compagnon

Isolation of *Clavibacter michiganensis* subsp. *michiganensis* from tomato seedlings: From each stem sample, tissue sections were surface-disinfected with 70% ethanol, rinsed with sterile distilled water and homogenized in a sterilized mortar in a 0.85% NaCl solution. An aliquot of 0.1 mL from each suspension was spread on a triplicate plate of nutrient-broth-yeast extract agar medium (NBYA), as well as, on two semi-selective media SCM and YDC[13]. The Petri dishes were incubated at 26°C for 3-10 days. After incubation, suspected colonies of *Cmm* were purified by sub-culturing and re-streaking on the NBYA medium. If there is no growth of suspect colonies on semi-selective media, the stem sample in question is considered negative for *Clavibacter michiganensis* subsp. *michiganensis*[15]. At all identification steps, one reference *Cmm* strain (H195), previously isolated from a diseased tomato plant in the laboratory of Phytobacteriology, Agronomy and Veterinary Institute (IAV) Agadir Morocco, was used as positive control[6]. Biochemical characterization of suspected-*Cmm* colonies was performed by using 3% KOH and oxidase standard tests[16].

Identification by Bio-PCR: In addition to using morphological identification to verify the presence of *Cmm* in stem samples, a genotypic identification of all *Cmm*-suspected colonies was performed from DNA extracted directly from cell suspension (OD600 nm approximately 0.05) obtained from *in-vitro*-grown pure bacterial strains on YDC-medium (not older than 5 days). A test tube with a cell suspension of the reference *Cmm* strain (H195) was used as a positive control. The suspensions were stored at -20°C until the PCR analysis. The DNA was extracted by using the DNeasy kit (Bioline, Meridian Bioscience), according to the protocol recommended by the manufacturer.

Polymerase Chain Reaction (PCR) amplification with specific primers was done according to the International Seed Federation protocol[13]. The PCR was carried out using primers RZ-ptssk 10: 5'-GGG GCC GAA GGT GCT GGT G-3' and RZ-ptssk 11: 5'-CGT CGC CCG CCC GCT G-3' and Wu Forward 5'-CAA CGC GAA GAA CCT TAC C-3' and Wu reverse 5'-ACG TCA TCC CCA CCT TCC-3'[13,17]. PCR conditions were: initial denaturation at 95° C for 5 min, the addition of 1 unit of Taq polymerase (MyTaq, Bioline, Meridian Bioscience) followed by 40 cycles of denaturation at 95°C for 15 sec, primer annealing at 60°C for the 30 sec and DNA extension at 72°C for 2 min. A final extension was completed at 72°C for 7 min. The amplified fragment was then purified by gel-chromatography in 2% agarose and afterwards stained using ethidium bromide[13].

Pathogenicity test: The Pathogenicity tests were carried out by using the cotyledons test[9,18]. The Cotyledons test was conducted with 4 days old tomato seedlings, at the first true leaf stage, the stems of tomato seedlings were cut, just above the cotyledons, with a disinfected scalpel blade preliminary immersed in one suspension (10^8 CFU/mL) of Bio-PCR assay-confirmed colonies. The inoculated seedlings were incubated in a growth chamber at 25-27°C. The treatments were carried out with the four suspect bacteria and the whole experiment was repeated three times. The negative controls consisted of seedlings inoculated with sterilized distilled water and the positive controls consisted of those inoculated with the pathogen alone (strain H195). The cotyledons were then examined for disease symptoms 5-7 days after inoculation. Positive test results in raised white blisters or cracked areas; In the event of a negative test, the cotyledons will remain well developed[18]. Twelve seedlings (24 cotyledons) were used for each bacterium and were arranged in three random blocks. The severity and virulence of each suspected bacterium were estimated by determining the percentage of seedlings with withered and desiccated cotyledons.

RESULTS

Isolation of *Cmm* from tomato seedlings samples: From more than 1000 bacteriological analyses (Fig. 1) realized during 3 years of the survey (2018, 2019 and 2020), 15 suspected-*Cmm* colonies were isolated from seedling samples becoming from different seed lots. Indeed, stem samples collected from the different scion, rootstock and grafted seedling (Table 1), allowed the isolation of *Cmm*-suspected colonies with uniform morphology in the used culture media NBYA, YDC and SCM (Fig. 2). For NBYA and YDC medium, 3 days incubation period was required to obtain

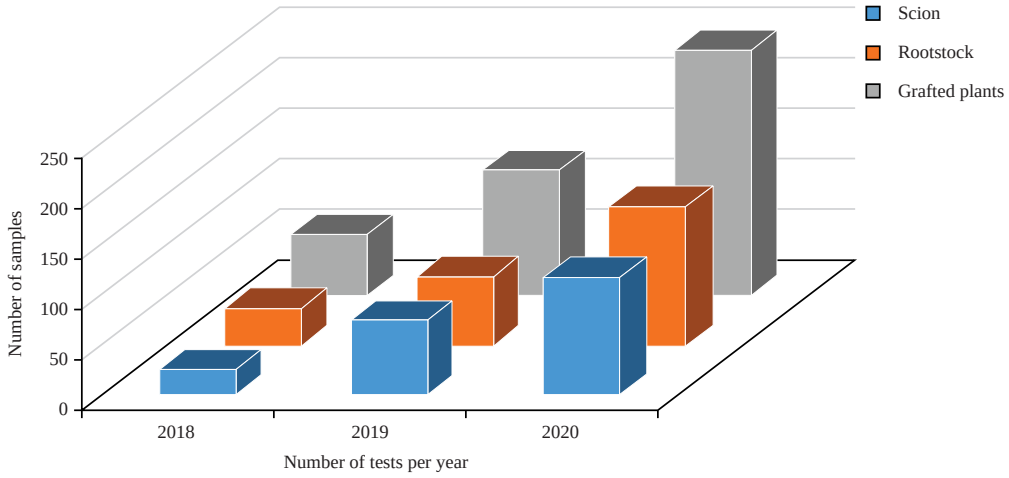

Fig. 1: Number of tests realized during 3 years of survey

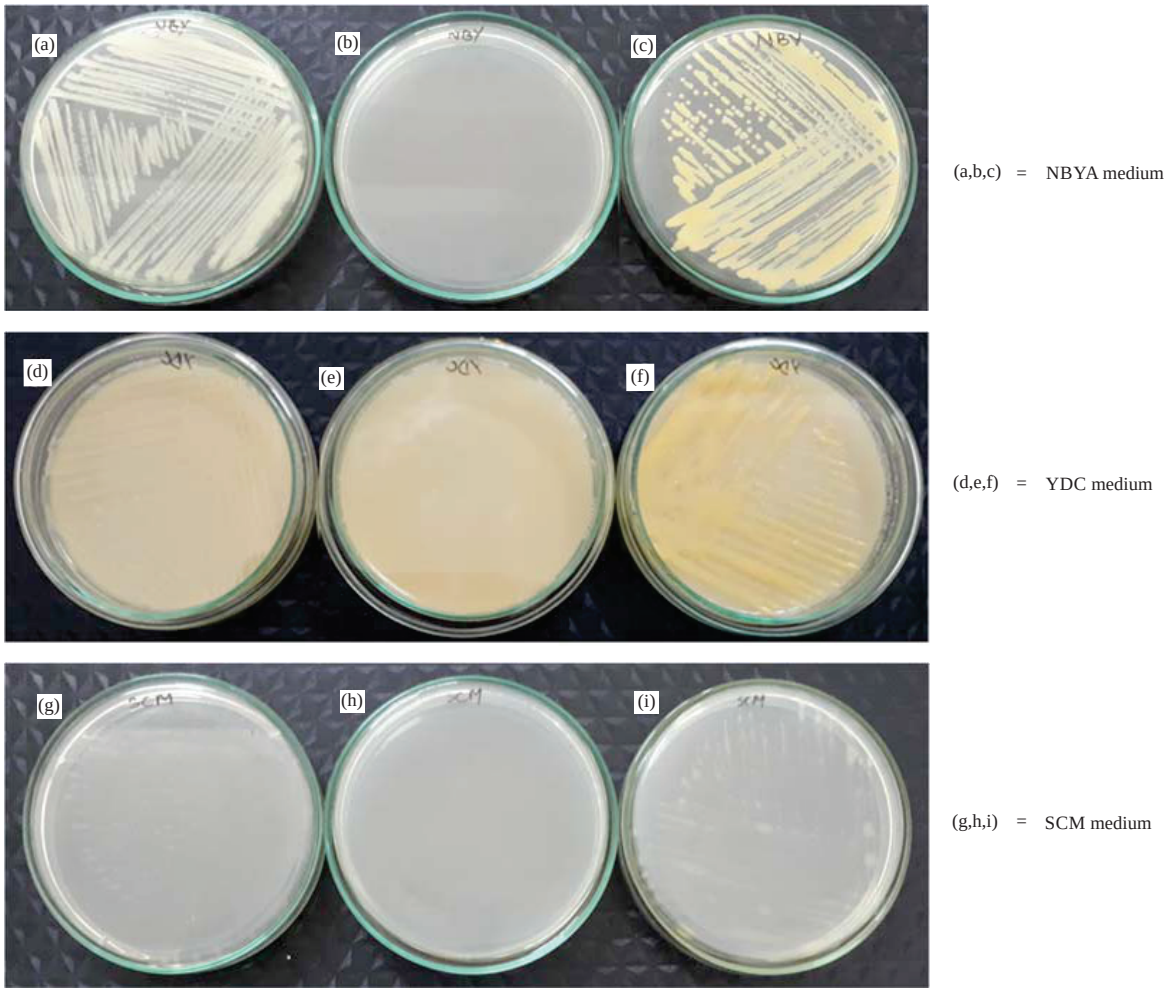

Fig. 2(a-i): Isolation of *Clavibacter michiganensis* subsp. *michiganensis* from seedling samples on culture media, (a, d, g) Seedling samples, (b, e, h) Negative control and (c, f, i) Positive control

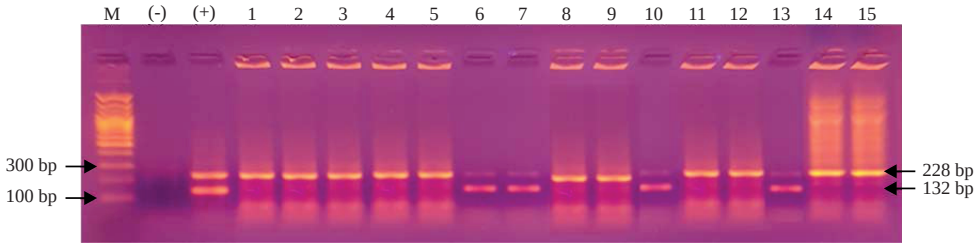

Fig. 3: Gel electrophoresis of amplicons after PCR
A 132 bp band was detected from four bacterial colonies (6, 7, 10, 13) showing the bacteria were *Clavibacter michiganensis* subsp. *michiganensis* from tomato seedlings, M: DNA size marker, (-): Negative control, (+): Positive control and Lanes 1-15: bacterial samples

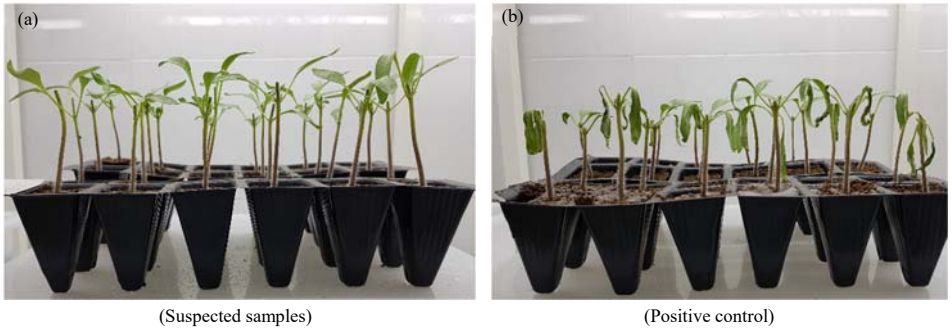

Fig. 4(a-b): Cotyledon tests were carried out on tomato seedlings by (a) Using four suspected bacterial samples and (b) *Cmm* H195 strain as a positive control

clear colonies to be counted, while for SCM medium, 7 days development was required. In positive control, the morphology of *Cmm* colonies was observable on NBYA, YDC and SCM. The colonies were yellow and mucoid on NBYA and YDC, in SCM the colonies were circular specked grey to black colour, convex, irregular and mucoid (Fig. 2a-i).

Biochemical identification of suspected colonies: For a preliminary identification of suspected-*Cmm,* all suspected isolates were subjected to KOH and oxidase biochemical tests. The results obtained revealed that all the suspected-*Cmm* isolates were oxidase positive and KOH test negative (gram-positive bacteria).

Molecular analysis: According to the results obtained, 15 suspected-*Cmm* isolates were isolated from seedling samples and 11 suspected-*Cmm* isolates didn't react with the primers, which gave negative results in PCR reactions. On the other hand, four suspected-*Cmm* isolates (lanes 6, 7, 10 and 13) have been amplified by PCR with the primers described above and produced an amplicon of size 132 bp (Fig. 3). Since false positives and false negatives can still be found using PCR, therefore final confirmation by Pathogenicity Testing is required.

Pathogenicity tests (Cotyledon test): The Cotyledon test is a simple and rapid *in vivo* test to confirm the pathogenicity of suspected *Cmm* isolates. The obtained results showed that the seedlings inoculated with the suspensions of the four *Cmm*-suspected isolates did not establish typical bacterial canker symptoms on inoculated tomato seedlings at 3-4 days post-inoculation (Fig. 4a-b). On the other hand, the positive control (*Cmm* strain H195) induced a typical collapse of the cotyledons 3 days after inoculation, while in the case of the negative control (sterile distilled water), the cotyledons expanded well developed with the development of new buds (Fig. 4).

DISCUSSION

The present study described the impact of the implementation of Good Seed and Plant Practices (GSPP) system, in a commercial tomato nursery, on the incidence of bacterial canker. Contaminated or infected tomato transplants by the *C. michiganensis* subsp. *michiganensis* are often considered to be the primary source of inoculum for bacterial canker transmission from nurseries to commercial tomato greenhouses. Moreover, bacterial canker symptoms were not observed on tomato seedlings before transplanting them into

tomato greenhouses. Therefore, the detection of symptomless infected tomato transplants in the nursery is the key to the control of tomato bacterial canker dissemination. According to Huang and Tu[10], 0.01-0.05% of contaminated tomato seeds or transplants can cause an epidemic under favourable greenhouse conditions. As a result, several commercial tomato nurseries in Morocco adopted a preventative system to minimize the hazard of contamination of tomato transplants by *Cmm*, during the seedlings production chain, through the implementation of the GSPP system. In the present study, two media (YDC and SCM) were used to detect *Cmm* in samples of asymptomatic tomato seedlings produced according to GSPP practice. The microbiological results indicated that *Cmm* was very low in all analysed samples during the 3 years of the survey. Indeed, the results obtained showed that from more than 1000 bacteriological analyses realized by dilution plating on semi-selective media recommended by the EPPO[4] and by the ISHI[19], only 15 suspected-*Cmm* colonies were isolated from seedling samples becoming from different seed lots. These results could be explained partially because the nursery had formal good seed and plant practices implemented, technical support, water control, inspections and a sampling plan for microbiological and molecular analyses. However, the sensitivity of the microbiological methods depends on several factors, such as the variation in the levels of seedlings contamination or infection, procedures used to extract bacteria from stem samples and infection rates of seedlings[20,21]. In a tomato nursery, a low number of contaminated or infected source seedlings may give rise to a large number of infected transplants even when careful measures are taken. According to Hausbeck *et al.*[22], the pathogen population must be reduced below 10^7 CFU/g of tissue at the time of transplanting to prevent yield loss in commercial tomato greenhouses. Usually, contaminated tomato seeds, poor hygienic conditions and/or contaminated workers were the most likely sources of the dispersal of *Cmm* in commercial tomato nurseries.

According to Schaad *et al.*[23], *Cmm* strains exhibit broad variation in colony morphology, even on the same culture medium. Therefore, it is recommended to use several techniques to confirm the presence of *Cmm* in symptomless tomato plantlets. The 21 suspected isolates identified as *Cmm* by using conventional microbiological methods were further analyzed using molecular methods. The obtained results demonstrated that the only a minority of the microbiological results corresponded with those of molecular, except for 4 out of 15, eleven of the analyzed seedlings samples were positive using the microbiological tests but negative for the PCR technique. In this study, the PCR amplifications were carried out with primers RZ-ptssk 10 and RZ-ptssk 11, according to the International Seed Federation protocol[13] and yielded an amplicon size of 132 bp for *Clavibacter michiganensis* subsp. *michiganensis*[24,25]. The Bio-PCR was performed for each *Cmm* suspected colony isolated from symptomless tomato seedlings. This technique is recommended for detecting *Cmm* in tomato seeds or transplants[20,26]. Also, detect the bacterial cells in extremely low levels in seeds and transplants[27] and detect the spread of *Cmm* bacteria in seedlings at the early stages of infection[28,29]. Previous studies have shown that the Bio-PCR is a sensitive and reliable technic for detecting *Cmm*[28,29] from seeds and transplants. However, this method does not quantify *Cmm* levels in the seedling samples, it only shows whether this pathogen is present or not.

In this study, the identification of *Cmm* suspected colonies were done according to their morphology on different culture media, Gram reaction, oxidase test and Bio-PCR Test. However, when a positive result is obtained, confirmation by pathogenicity test should be performed to conclude the infection of the tomato seedlings by *C. michiganensis* subsp. *michiganensis*[4,13].

After the Bio-PCR analysis, the four suspected isolates and reference strain of *C. michiganensis* subsp. *michiganensis* were further tested for their pathogenicity with the cotyledons tests. The appearance of typical bacterial canker symptoms is related to the virulence of the bacteria tested. After four days, the cotyledons of the inoculated tomato seedlings did not show clear symptoms of infection by *C. michiganensis* subsp. *michiganensis*[28], while the positive control showed the typical collapse of the cotyledons. The cotyledon test was used to distinguish *C. michiganensis* subsp. *michiganensis* from other gram-positive bacteria with similar cultural characteristics and to determine the virulence of each *Cmm* suspected isolates[13,25,30].

The results of this study highlight the impact of the implementation of the GSPP system on bacterial canker occurrence in a commercial tomato-seedlings nursery. Furthermore, the successful control of this bacterial disease in tomato-seedling nurseries could have a significant impact on the overall incidence rate of bacterial canker in tomato commercial production greenhouses.

CONCLUSION

Microbiological and molecular results indicated that *Clavibacter michiganensis* subsp. *michiganensis* was absent in all analysed tomato seedlings samples and no infected or contaminated seedlings were detected during the 3 years of

the survey. These results demonstrated that the rigorous application of good seed and plant practices by the tomato nursery, as well as the strict hygiene measures, could reduce the risk of contamination of the grafted tomato seedlings by the bacterium. To the best of our knowledge, it is the first work carried out to provide information on the incidence of bacterial canker in tomato seedlings produced in a commercial nursery under GSPP accreditation.

SIGNIFICANCE STATEMENT

The purpose of this work is to study the impact of the application of the Good Seed and Plant Practices (GSPP) standard on the quality of tomato seedlings to bacterial canker. This work shows that the application of GSPP by the tomato nursery could reduce or avoid the risk of contamination of the grafted tomato seedlings by *Clavibacter michiganensis* subsp. *michiganensis*. Indeed, the detection of symptomless infected tomato seedlings in commercial nurseries is the key to the control of bacterial canker in the tomato production chain.

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REFERENCES

1. Ftayeh, R., A. von Tiedemann, B. Koopmann, K. Rudolph and M. Abu-Ghorrah 2008. First record of *Clavibacter michiganensis* subsp. *michiganensis* causing canker of tomato plants in Syria. Plant Dis., 92: 649-649.
2. Wang, Y., S. Deng, Z. Li and W. Yang, 2022. Advances in the characterization of the mechanism underlying bacterial canker development and tomato plant resistance. Horticulturae, Vol. 8. 10.3390/horticulturae8030209.
3. Kleitman, F., I. Barash, A. Burger, N. Iraki and Y. Falah *et al*., 2008. Characterization of a *Clavibacter michiganensis* subsp. *michiganensis* population in Israel. Eur. J. Plant Pathol., 121: 463-475.
4. European and Mediterranean Plant Protection Organization, 2016. PM 7/42 (3) *Clavibacter michiganensis* subsp. *michiganensis*. Bull. OEPP/EPPO Bull., 46: 202-225.
5. Bella, P., G. Ialacci, G. Licciardello, R.L. Rosa and V. Catara, 2012. Characterization of atypical *Clavibacter michiganensis* subsp. *michiganensis* populations in greenhouse tomatoes in Italy. J. Plant Pathol., 94: 635-642.
6. Boudyach, E.H., M. Fatmi, H. Boubaker, A.A.B. Aoumar and O. Akhayat, 2004. Effectiveness of fluorescent pseudomonads strains HF22 and HF142 to control bacterial canker of tomato. J. Food Agric. Environ., 1: 115-120.
7. Fatmi, M. and N.W. Schaad, 2002. Survival of *Clavibacter michiganensis* ssp. *michiganensis* in infected tomato stems under natural field conditions in California, Ohio and Morocco. Plant Pathol., 51: 149-154.
8. Daferera, D.J., B.N. Ziogas and M.G. Polissiou, 2003. The effectiveness of plant essential oils on the growth of *Botrytis cinerea, Fusarium* sp. and *Clavibacter michiganensis* subsp. *michiganensis*. Crop Prot., 22: 39-44.
9. Amkraz, N., E.H. Boudyach, H. Boubaker and A.A.B. Aoumar, 2010. Screening for fluorescent pseudomonades, isolated from the rhizosphere of tomato, for antagonistic activity toward *Clavibacter michiganensis* subsp. *michiganensis*. World J. Microbiol. Biotechnol., 26: 1059-1065.
10. Huang, R. and J.C. Tu, 2001. Effects of nutrient solution pH on the survival and transmission of *Clavibacter michiganensis* ssp. *michiganensis* in hydroponically grown tomatoes. Plant Pathol., 50: 503-508.
11. Werner, N.A., D.W. Fulbright, R. Podolsky, J. Bell and M.K. Hausbeck, 2002. Limiting populations and spread of *Clavibacter michiganensis* subsp. *michiganensis* on seedling tomatoes in the greenhouse. Plant Dis., 86: 535-542.
12. FAO/IPPC 2017. The use of integrated measures in a systems approach for pest risk management. ISPM No. 14 (Rome: FAO). https://www.fao.org/3/y4221e/Y4221E.pdf.
13. Fatmi, M., H. Bolkan and N.W. Schaad, 2019. Detection of *Clavibacter michiganensis* subsp. *michiganensis* in Tomato Seeds. In: Detection of Plant-Pathogenic Bacteria in Seed and Other Planting Material, Fatmi, M., R.R. Walcott and N.W. Schaad (Eds.), The American Phytopathological Society, Saint Paul, Minnesota, USA, ISBN: 978-0-89054-541-6, pp: 111-117.
14. Xu, X., S.A. Miller, F. Baysal-Gurel, K.H. Gartemann, R. Eichenlaub and G. Rajashekara, 2010. Bioluminescence imaging of *Clavibacter michiganensis* subsp. *michiganensis* infection of tomato seeds and plants. Appl. Environ. Microbiol., 76: 3978-3988.
15. de León, L., F. Siverio, M.M. López and A. Rodríguez, 2008. Comparative efficiency of chemical compounds for *in vitro* and *in vivo* activity against *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of tomato bacterial canker. Crop Prot., 27: 1277-1283.
16. Kaneshiro, W.S., C.Y. Mizumoto and A.M. Alvarez, 2006. Differentiation of *Clavibacter michiganensis* subsp. *michiganensis* from seed-borne saprophytes using ELISA, Biolog and 16S rDNA sequencing. Eur. J. Plant Pathol., 116: 45-56.
17. Bach, H.J., I. Jessen, M. Schloter and J.C. Munch, 2003. A TaqMan-PCR protocol for quantification and differentiation of the phytopathogenic *Clavibacter michiganensis* subspecies. J. Microbiol. Methods, 52: 85-91.

18. Tripathi, R., K. Vishunavat and R. Tewari, 2022. Morphological and molecular characterization of *Clavibacter michiganensis* subsp. *michiganensis* causing bacterial canker in tomatoes. Physiol. Mol. Plant Pathol., Vol. 119. 10.1016/j.pmpp.2022.101833.
19. Fatmi, M., R.R. Walcott and N.W. Schaad, 2017. Detection of Plant-Pathogenic Bacteria in Seed and Other Planting Material. 2nd Edn., The American Phytopathological Society, Saint Paul, Minnesota, USA, ISBN: 978-0-89054-541-6, Pages: 360.
20. Hadas, R., G. Kritzman, F. Klietman, T. Gefen and S. Manulis, 2005. Comparison of extraction procedures and determination of the detection threshold for *Clavibacter michiganensis* ssp. *michiganensis* in tomato seeds. Plant Pathol., 54: 643-649.
21. Ftayeh, R.M., A. von Tiedemann and K.W.E. Rudolph, 2011. A new selective medium for isolation of *Clavibacter michiganensis* subsp. *michiganensis* from tomato plants and seed. Phytopathology, 101: 1355-1364.
22. Hausbeck, M.K., J. Bell, C. Medina-Mora, R. Podolsky and D.W. Fulbright, 2000. Effect of bactericides on population sizes and spread of *Clavibacter michiganensis* subsp. *michiganensis* on tomatoes in the greenhouse and on disease development and crop yield in the field. Phytopathology®, 90: 38-44.
23. Schaad, N.W., J.B. Jones and W. Chun, 2001. Laboratory Guide for Identification of Plant Pathogenic Bacteria. 3rd Edn., APS Press, Saint Paul, Minnesota, USA, ISBN: 9780890542637, Pages: 373.
24. Croce, V., M.J. Pianzzola, K. Durand, M. González-Arcos, M.A. Jacques and M.I. Siri, 2016. Multilocus sequence typing reveals high variability among *Clavibacter michiganensis* subsp. *michiganensis* strains affecting tomato crops in Uruguay. Eur. J. Plant Pathol., 144: 1-13.
25. Gartemann, K.H., O. Kirchner, J. Engemann, I. Gräfen, R. Eichenlaub and A. Burger, 2003. *Clavibacter michiganensis* subsp. *michiganensis*: First steps in the understanding of virulence of a gram-positive phytopathogenic bacterium. J. Biotechnol., 106: 179-191.
26. de León, L., F. Siverio, M.M. López and A. Rodríguez, 2011. *Clavibacter michiganesis* subsp. *michiganensis*, a seedborne tomato pathogen: Healthy seeds are still the goal. Plant Dis., 95: 1328-1338.
27. Schaad, N.W., Y. Berthier-Schaad and D. Knorr, 2007. A high throughput membrane BIO-PCR technique for ultra-sensitive detection of *Pseudomonas syringae* pv. *Phaseolicola*. Plant Pathol., 56: 1-8.
28. Burokienė, D., 2006. Early detection of *Clavibacter michiganensis* subsp. *michiganensi* in tomato seedlings. Agron. Res., 4: 151-154.
29. Sen, Y., J. van der Wolf, R.G.F. Visser and S. van Heusden, 2015. Bacterial canker of tomato: Current knowledge of detection, management, resistance, and interactions. Plant Dis., 99: 4-13.
30. Bibi, A., M. Junaid and M. Ahmad, 2018. Pathogenicity and inoculum concentration effects of *Clavibacter michiganensis* subsp. *michiganensis* on severity of bacterial canker of tomato. Novel Res. Microbiol. J., 2: 167-174.