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# Occurrence of *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of bacterial canker of tomato, in Syria

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**Summary.** Several surveys were carried out to evaluate the occurrence of bacterial canker of tomato caused by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) in Syria, especially in the North-West provinces Latakia and Tartous. The surveys revealed typical disease symptoms in greenhouses where the tomato cvs. Dima, Huda and Astona were grown, such as dark brown to black lesions on the leaf margins, wilting of whole plants, stunting, and vascular discoloration. The disease incidence in such greenhouses was 15% in the spring of 2007, and up to 70% by the end of July. Ten isolates obtained from diseased plants at different locations in these two provinces were identified as *Clavibacter michiganensis* subsp. *michiganensis* using classical microbiological tests as well as PCR. This is the first detailed proof of the occurrence of bacterial canker of tomato in Syria.

**Key words**: *Solanum lycopersicum* L., survey, first record, pathogenicity.

## Introduction

*Clavibacter michiganensis* subsp. *michiganensis* (Smith, 1910) Davis *et al*., 1984 (*Cmm*) causes one of the most serious and injurious bacterial diseases of tomato (*Solanum lycopersicum* L.). It is listed as an A2 quarantine pathogen by EPPO and now occurs in many tomato-growing areas worldwide, including the EPPO region (EPPO/CABI, 1998) and many neighbouring countries. In Syria too *Cmm* is a quarantine organism and imported tomato seeds must be free of this pathogen. So far the occurrence of the disease in Syria has not been comprehensively studied, apart from one abstract (Ftayeh *et al*., 2008). The bacterium causes yield losses of up to 60% (Griesbach *et al*., 2000) and it has several alternative host plants, such as *Capsicum annuum, Solanum melongena*, *S. nigrum* and *S. triflorum* (Strider, 1969). The pathogen survives in seeds, on greenhouse structures, in plant debris (Strider, 1969; Fatmi & Schaad, 2002), and to a certain extent in soil (Ftayeh *et al*., 2004).

Contaminated seeds and young plants are the principal means for long-distance transmission of the pathogen (Strider, 1969). A minute number of contaminated seeds (1–5 in 10.000) can cause an epidemic in field-grown tomatoes (Chang *et al*., 1991; Gitaitis *et al.,* 1991). Even symptomless tomato seedlings may harbour high populations of *Cmm* (Werner *et al*., 2002) and infect other tomato plants later. Since there are as yet no effective bactericides, or high-yielding *Cmm*-resistant tomato cultivars available (Boelema, 1980), strict hygienic measures are currently the only way to control the disease. Most important is the use of pathogen-free tomato seeds.

The aim of this study was to survey bacterial canker of tomato in the Syrian provinces Latakia

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Table 1. Areas and yield of tomatoes in Syria and in the Syrian provinces Latakia and Tartous in 2007 (Anonymous, 2007).

| Tomato production | Open field | | | Greenhouse | | |
|---|---|---|---|---|---|---|
| | Syria | Latakia province | Tartous province | Syria | Latakia province | Tartous province |
| Area (ha) | 15235 | 677 | 404 | 3759 | 418 | 3287 |
| Yield (ton) | 731251 | 13440 | 6371 | 501204 | 55740 | 438300 |

and Tartous along the Mediterranean Sea, where almost all Syrian greenhouse tomatoes are grown, destined for the Syrian market in winter and for export.

## Materials and methods

### Surveys and sample collection

Between March and mid-April of 2007, and again at the end of July 2007, a number of surveys were carried out in greenhouses (plastic tunnels 2.5–3.0 m in height) in Latakia and Tartous along the Mediterranean Sea in North-West Syria (Figure 1), where 82,340 greenhouses were cultivated with tomatoes in 2007 (Table 1). One hundred and fifty greenhouses with a total acreage of 6,75 ha were surveyed. Most of the greenhouses were randomly selected but a few were chosen because local agricultural advisers observed wilt symptoms in them. Disease incidence caused by *Cmm* in these greenhouses was estimated by dividing the number of plants with wilt symptoms by the total number of plants in the greenhouse. From each greenhouse with disease occurrence, stem samples of wilted tomato plants were taken and stored under cool conditions until isolating the causal organism.

Figure 1. Intensive greenhouse tomato cultivation in the coastal Mediterranean provinces of Syria.

**Isolation and identification**

Bacterial isolates were obtained and purified in the laboratory of the Plant Protection Directorate in Damascus, Syria, and all further laboratory tests were conducted at the Division of Plant Pathology and Crop Protection, University of Göttingen, Germany. Stem samples from diseased plants were surface-disinfected with 70% ethanol and homogenized in a sterilized mortar in sterile water. Serial dilutions (1:10) until $10^{-5}$ of the homogenate were made in 0.01M $MgSO_4$, and 0.1 mL from each dilution was plated on nutrient glucose agar medium (NGA) containing 0.8% nutrient broth, 1% glucose, 0.3% yeast extract (Mavridis, University of Göttingen, Germany: personal communication), as well as on a new semiselective medium for *Cmm* still being developed. The Petri dishes were incubated at 26°C and evaluated after 3 or 5 days on NGA or the new semiselective medium, respectively.

Putative colonies of *Cmm* were purified by sub-culturing and repeated re-streaking on Petri dishes containing NGA. Isolates were initially identified on the basis of colony characteristics and cell morphology (colour, shape, motility and size), Gram's reaction with 3% KOH (Gregersen, 1978), and a hypersensitive reaction on the leaves of four o'clock plants (*Mirabilis jalapa*) (Gitaitis, 1990) using bacterial suspensions of $10^8$ cfu $mL^{-1}$ prepared photometrically (Spectronic 20, Bausch & Lomb Inc., Rochester, NY, USA). Final identification of the isolates was confirmed by both PCR and pathogenicity tests. At all identification steps, two reference *Cmm* strains (2973 and 390) obtained from the Göttinger Sammlung Phytopathogener Bakterien (GSPB), were used as positive controls. As negative controls, plants were inoculated with 0.01M sterile $MgSO_4$ solution.

**Pathogenicity**

Pathogenicity of the isolates was tested by mechanically inoculating 6-week-old tomato plants (cv. Lyconorma). Each isolate and strain was inoculated into three tomato seedlings. The inoculum was prepared by suspending a loopful of a 24-h-old bacterial culture grown on NGA in 0.01M of sterile $MgSO_4$, and the suspension was adjusted to an optical density of 0.06 at 660 nm (Spectronic 20, Bausch & Lomb Inc.) corresponding to about $10^8$ cfu $mL^{-1}$. A 35 $\mu$L drop was placed in the axil of the second or third true leaf (Mavridis *et al.*, 1990). Inoculation was performed by pricking the stem through the drop with a sterile needle. For the negative control, the tomato seedlings were inoculated with sterile 0.01M $MgSO_4$. The plants were kept at room temperature (18°C) for 12 h and later in a glasshouse at 25/20°C (day/night) with a relative humidity between 50 and 90%. Plants were checked regularly for symptom development.

Symptoms were recorded within 10 to 15 days after inoculation. To fulfil Koch´s postulates, the pathogen was re-isolated and re-identified from the inoculated plants showing disease symptoms.

**PCR identification**

DNA of all *Cmm* strains was isolated from *in-vitro*-grown pure bacterial strains with the MasterPure Gram Positive DNA Purification Kit (Epicentre Biotechnologies, Madison, WI, USA). Concentrations of DNA were assessed after standard gel electrophoresis (1.2% w:v of agarose dissolved in 0.5% TBE-buffer, stained with 0.5 $\mu$g $mL^{-1}$ ethidium bromide, 3 v/cm, 120 min) in comparison with different concentrations of Lambda DNA (MBI Fermentas, St. Leon-Rot, Germany).

The polymerase chain reaction (PCR) was carried out using the specific primer set PSA-4 and PSA-R proposed by Pastrik and Rainey (1999). Amplification was performed in a total volume of 25 $\mu$L. The reaction mix contained 1× reaction buffer (10 mM Tris-HCl of pH 8.8 at 25°C, 50 mM KCl, 0.8% Nonidet P40) and was supplemented with 1.5 mM $MgCl_2$, 0.2 mM dNTP, 1 $\mu$M of each primer, 1 U Taq DNA polymerase (MBI Fermentas) and 1 ng of template DNA. The PCR profile consisted of an initial denaturation step at 95°C for 4 min, followed by 35 amplification cycles at 95°C for 1 min, 63°C for 1 min and 72°C for 1 min. The final elongation step was done at 72°C for 10 min. Amplification was performed using a PTC 100 thermo cycler (MJ Research, Watertown, MD, USA). PCR products and the GeneRuler™ 100 bp DNA ladder (MBI Fermentas) were separated on 1.5% agarose gel. Gels were stained in 0.5 $\mu$g $mL^{-1}$ ethidium bromide solution for 10 min.

Figure 2. Symptoms seen in greenhouses. A. Discoloration of leaf margins. B. Wilting of whole plants.

## Results

### Disease incidence

Typical symptoms of bacterial canker were found in 10 of the 150 greenhouses. Symptoms such as stunting, dark brown-to-black lesions on the leaf margins (Figure 2a), and vascular discoloration followed by wilting (Figure 2b) were seen on the tomato cultivary. Dima, Huda and Astona. Disease incidence in these greenhouses was estimated at up to 15% by the middle of April 2007. By the end of July, it had increased to a maximum of 70% in two of these greenhouses, to 30–40% in 6 greenhouses, and was still 15% in the remaining two greenhouses. Obviously, disease incidence varied depending on how actively farmers destroyed infected and adjacent plants and followed the recommended hygienic measures. In 2008 and 2009 no surveys were conducted. Wilt symptoms were seen by agricultural advisers (M. Eshbani) in

some greenhouses, but laboratory tests for isolation of the causal pathogen were not done.

**Isolate identification**

Ten bacterial isolates, subsequently identified as *Clavibacter michiganensis* subsp. *michiganensis,* were obtained from various greenhouses at different locations in both provinces: from Ayn Erraheb and Bostan Eljamee in Latakia, and from Banyas, Hryson and Alkhrab Alshamali in Tartous. Three days after streaking these strains onto NGA and 5 days after streaking them on the new semiselective medium, typical *Cmm* colonies appeared when incubated at 26°C. Colonies were 2 to 3 mm, light yellow, brilliant, convex and slimy, round or with irregular margins. *Cmm* colonies on NGA and on the new medium were very similar. However, the new medium strongly suppressed saprophytic bacteria.

Microscopically, the bacterial cells were coryneform in shape and non-motile. All the isolates were Gram-positive and induced hypersensitive reactions on four o'clock plants (*Mirabilis jalapa*) within 24 h after inoculation.

**Pathogenicity**

All the isolates and the reference strains induced the typical symptoms of bacterial canker on mechanically inoculated young tomato plants in 10 to 15 days. These symptoms included unilateral wilt of leaflets (Figure 3a) and cankers on the stems (Figure 3b) followed finally by wilting of entire plants. Control plants inoculated with 0.01M $MgSO_4$ solution did not show any symptoms. In order to fulfil Koch's postulates, re-isolation and re-identification of the pathogen was performed from these artificially inoculated plants.

**PCR identification**

Amplifications using the primer pair PSA-4 and PSA-R produced the expected amplicons of 270 bp with both the two reference strains and all the 10 Syrian isolates (Figure 4), as described by Pastrik and Rainey (1999).

## Discussion

Bacterial canker of tomato has not been reported before in Syria (Ftayeh *et al*., 2008). Similar symptoms such as stunting or wilting of tomato plants and discoloration of the vascular system may have been seen in the past but they were not further investigated in Syria, probably because they were mistaken for Fusarium wilt (M. Eshbani, personal communication). In addition, the exchange of information between Syria and the EPPO was not very intensive in the past. This is therefore the first detailed report and confirmation of bacterial canker occurring on tomatoes in Syria.

Although the total yield of greenhouse grown tomatoes in Syria is lower than that of field tomatoes (Table 1), greenhouse tomatoes are economically very important because they are harvested in winter and represent the only source of fresh tomatoes in winter for the market in Syria, and they are also exported. The price of fresh tomatoes is much high-

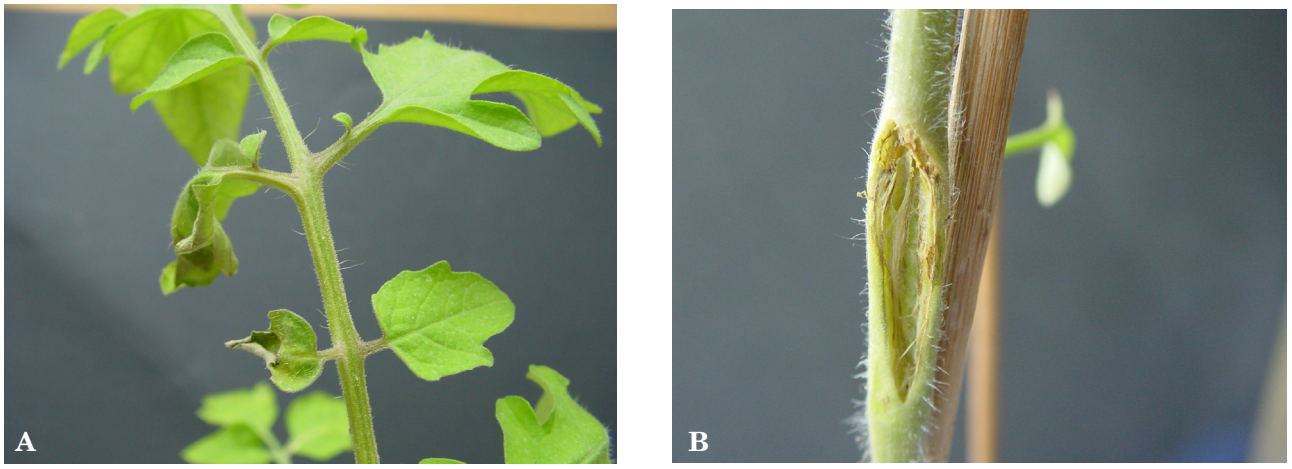


Figure 3. Symptoms seen after inoculation. A. Unilateral wilt of leaflets. B. Canker on tomato stem.

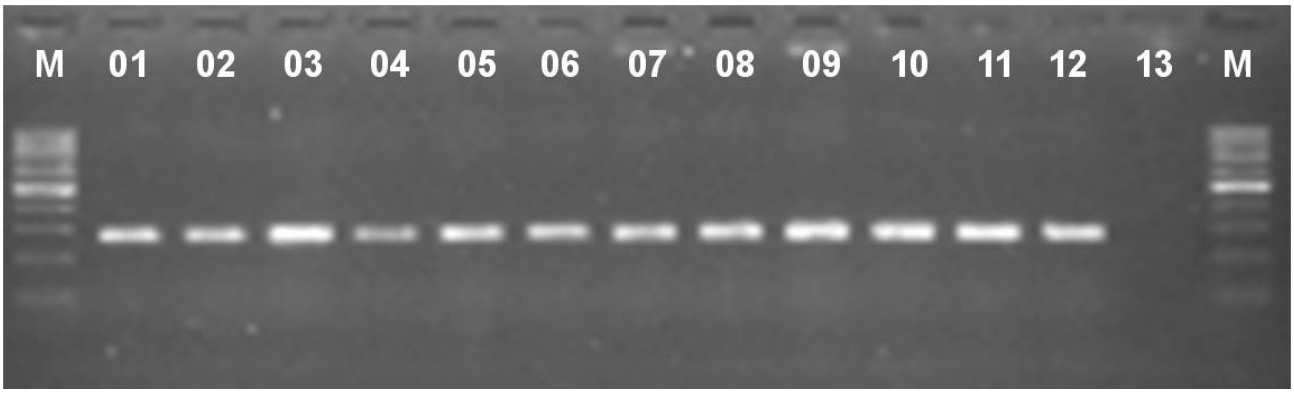


Figure 4. Gel electrophoresis of amplicons after PCR. M, GeneRuler™ 100bp DNA ladder; 01–10, Syrian *Cmm* isolates; 11 and 12, positive controls (*Cmm* GSPB 2973 and 390); 13, negative control (water).

er in winter than in summer. Consequently this study focused on greenhouse tomatoes.

The economic losses caused by *Cmm* in this part of the country can only roughly be estimated. In the surveys, 150 greenhouses in Latakia and Tartous out of 82,340 existing greenhouses (Anonymous 2007), or only 0.18% of the total, were carefully inspected for *Cmm*. Ten infected greenhouses out of 150 signifies an infection rate of 6.6%. However, since some of the greenhouses examined were not selected at random but on the basis of information provided by agricultural advisers, it is assumed that overall only 2% of all greenhouses were infected with *Cmm;* or 1,647 greenhouses. In this part of Syria the average yield of tomatoes per greenhouse is 6 t (Table 1), so that a loss of 20% from *Cmm* would amount to 1.2 t per greenhouse, or 2000 t for all infected greenhouses. Assuming a wholesale selling-price of 0.50 € per kg for the farmer and a retail market price of 1.00 € per kg, this would signify that *Cmm* caused an economic loss of 1 million € to farmers and a loss of 2 million € on the market.

Discussions with Syrian farmers and agricultural advisers revealed that bacterial canker had not been noticed in this part of the country before. The typical symptoms were certainly not detected in the year before the present survey was initiated in any of the greenhouses later found to be infected in the survey. This suggests that the pathogen may have been introduced recently by infected or contaminated seeds, although the seed from which the diseased tomato plants were grown had been certified as healthy. The survey also found that the disease did not turn into an epidemic. Instead, disease incidences occurred in diverse locations in both provinces Latakia and Tartous, obviously scattered all over this region. These findings also suggest that bacterial canker when it occurred derived from a very few and only slightly infested tomato seeds which remained undetected in the tomato seed lots that are regularly imported from overseas. It is therefore strongly recommended that in future all lots of tomato seeds and young plants should be carefully inspected for latent infection or contamination by *Cmm* before permitting them to enter the country.

After the survey was completed, some recommendations were given to Syrian farmers to help them manage bacterial canker and avoid further infections. The recommendations were: to destroy all infected and adjacent plants together with their root system, to disinfect all cutting tools with 70% ethanol, not to exchange or move tools between greenhouses, and to make all workers aware of the symptoms of bacterial canker. It is vital to eradicate all plants with their main root systems at the end of the vegetation period. When severe outbreaks of bacterial canker occur, the soil should be damped or solarized if possible. And in any case, it is strongly recommended to use certified healthy seeds every year.

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