

# Screening for new sources of resistance to *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) in tomato

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**Abstract** Bacterial canker of tomato, caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), is considered the most serious bacterial threat, resulting in high damages in production areas. World-wide, Cmm is subjected to quarantine regulations. There is no cultivar in market containing Cmm resistance genes. This project aimed to screen tomatoes or wild relatives of tomato for resistance to Cmm, to be used for starting breeding programs. We have screened 24 different wild accessions of tomato and found several new tolerant sources: *Solanum pimpinellifolium* GI.1554, *S. parviflorum* LA735 and *S. parviflorum* LA2072. We also confirmed the tolerance which was reported previously in *S. peruvianum* LA2157, *S. peruvianum* PI127829, *S. peruvianum* LA385, *S.*

*habrochaites* LA407 and *S. lycopersicum* cv. IRAT L3. No immunity was found. Also accessions showing a low disease score still contained high titers of bacteria as determined by a dilution plating method, using tow selective media. These results were confirmed with a TaqMan real time PCR assay, which was developed to determine and quantify Cmm *in planta*.

**Keywords** *Clavibacter* · Cmm · Tomato · TaqMan · Screening

## Introduction

Bacterial canker caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) was first described by Smith in 1910. This pathogen is considered the most serious bacterial disease of tomato. In artificially infected fields, the damage caused by Cmm can vary from 46 to 93 % (Poysa 1993). Also, this pathogen can cause high economic damage in commercial greenhouses. The pathogen is transmitted over long distances through seeds and spreads in the field due to cultural practices and physical contact of plants. Wounds, natural openings, such as hydathodes and stomata, and roots are the main ports of entrance for Cmm invasion of host tissues (Carlton et al. 1998). Once inside, the pathogen is translocated via xylem vessels throughout the plant. The spreading is unequal resulting in parts with or without the pathogen and also the concentration can vary. Unilateral wilting of

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leaves (one side wilting) is the first and typical symptom. Later stage symptoms can be severe stem canker and discoloration of vascular tissue. Severe symptoms are leaf necrosis which is often called ‘firing’ and dying of dark green colored plants due to water impairment. At late plant stage infection, there are no typical wilting symptoms but on fruits, black spots with a white halo (bird eyes) can be seen (Gartemann et al. 2003). This fruit infection can result in infected seeds and those are the main vectors for long distance spreading of the disease.

The first reported tolerant accession was *Solanum pimpinellifolium* in 1934 (Thyr 1976). Later, other tolerant accessions were reported such as *Solanum habrochaites* (Francis et al. 2001; Hassan et al. 1968; Sotirova and Achkova 1989; Sotirova et al. 1994), *Solanum lycopersicum* (Elenkov 1965; Emmatty and John 1973; Kuriyama and Kuniyasu 1974; Laterrot et al. 1978) and *Solanum peruvianum* (Lindhout and Purimahua 1989; Sotirova et al. 1994). Some of these tolerant sources were used for introgression breeding such as Bulgaria 12 (Elenkov 1965), Heinz 2990 (Emmatty and John 1973), Okitsu sozai 1–20 (Kuriyama and Kuniyasu 1974). These varieties were tolerant and showed partial wilting but still had a high bacterial titer. Tolerant sources, which were characterized by (van Heusden et al. 1999) and by (Francis et al. 2001), were also containing high bacterial levels. Breeding for Cmm tolerant cultivars is difficult due to the complex inheritance, both polygenic and additive.

Cmm is internationally a quarantine organism (Anonymous 2000) therefore an accurate detection is a crucial step in confirming the presence and preventing the spread of bacteria. Generally there is requirement for a fast, sensitive, highly specific, cheap and easy method. Different methods for detection have been described and each with their own advantages and disadvantages. Unfortunately there is no method that can meet all requirements and depending of its application, different methods or combinations of methods are used. Three different types of methods are in use: serological, DNA based and plating. Serological methods have a high risk of cross reaction with non-target organisms and the sensitivity of this method is low. Dilution plating on selective media is very sensitive but laborious and it takes 5–7 days to get results. DNA based methods, like TaqMan real time PCR assays, are fast, sensitive and highly specific

and it also allows quantification. TaqMan assay has been used to identify *Clavibacter* subspecies (Bach et al. 2003) and to identify Cmm in seeds (Zhao et al. 2007). DNA is a relatively stable molecule that can persist for a long time in the environment upon cell death. Therefore the TaqMan assay is not able to distinguish dead from viable bacteria. DNA from dead cells can be selectively removed during extraction by adding the DNA binding dye ethidium monoazide (EMA). EMA penetrates only dead cells with a comprised membrane, binds to its DNA and is covalently linked to the DNA with light. During the extraction the DNA–EMA complex will precipitate whereas the unbound DNA remains in solution. EMA was effective up until a concentration of  $10^8$  cfu/ml bacteria (Luo et al. 2008). Dead cells can also be distinguished from viable cells in a TaqMan based procedure by first plating the extract on a selective medium prior to TaqMan (Bio-TaqMan assay). Only viable bacteria that has formed to colonies will be detected. TaqMan assays have not been used for quantification of Cmm in planta yet.

In this study, we screened wild tomato gene resources for high resistance/tolerance and Cmm free material. The TaqMan assay was used to determine and quantify Cmm in a fast, easy and reliable way *in planta*.

## Materials and methods

### Plant material

Twenty four wild species of tomato (Table 1) including reported tolerance sources *S. peruvianum* LA2157, LA385, LA334, *S. habrochaites* LA407 and *S. lycopersicum* IRAT L3 were screened for tolerance to Cmm. *Solanum lycopersicum* cv. Moneymaker was added to the screening as susceptible control. Each accession was represented with 5 plants in the screening of which three were used for bacteria quantification.

### Disease test

The bacterial strain Cmm 542, which is known to be aggressive in tomato, was used for inoculation at the sixth leaf stage by removing the second leaf with scissors and injecting 5  $\mu$ l of  $10^8$  cfu/ml bacterial

**Table 1** Disease score and tolerance level of 25 tomato accessions

Accession name	Disease score	Tolerance level
<i>S. peruvianum</i> LA 2157	1	Group 1 (tolerant)
<i>S. pimpinellifolium</i> GI 1554	2	
<i>S. peruvianum</i> LA 385	2	Group 2 (moderate)
<i>S. lycopersicum</i> cv. IRAT L3	2	
<i>S. peruvianum</i> PI 127829	2	
<i>S. parviflorum</i> LA 735	2	
<i>S. parviflorum</i> LA 2072	2	
<i>S. glandulosum</i> IVT 63102	2	
<i>S. minutum</i> CGN 15816	2	
<i>S. glandulosum</i> EC 495	2	
<i>S. peruvianum</i> LA 2334	3	
<i>S. habrochaites</i> LA 407	3	
<i>S. habrochaites glabratum</i> GI 1561	3	Group 3 (susceptible)
<i>S. chilense</i> IVT 56140	3	
<i>S. glandulosum</i> IVT 48090	3	
<i>S. minutum</i> LA 1045	3	
<i>S. parviflorum</i> LA 2133	3	
<i>S. chilense</i> IVT 56139	4	
<i>S. lycopersicum</i> cv MoneyMaker	5	
<i>S. pennellii</i> LA 716	5	
<i>S. habrochaites</i> LYC4	5	
<i>S. cheesmanii</i> LA 1401	5	
<i>S. cheesmanii</i> LA 0166	5	
<i>S. cheesmanii</i> LA 1448	5	
<i>S. cheesmanii</i> LA 1409	5	

suspension in the wound. Approximately 10 cm above the first inoculation (between the fourth and fifth leaves), a second inoculation was done by injecting again 5 µl bacterial suspension. After inoculation, plants were kept for one week under high relative humidity (100 %) and subsequently at 60 % humidity, 12 h day light, 24 °C day and 18 °C night temperature. Symptoms of bacterial canker were recorded using the following scale: no symptoms, score = 0; 1 leaf wilting, score = 1; more than one leaf but <50 % leaves are wilted, score = 2; between 50 and 75 % leaves are wilted, score = 3; more than 75 % but not all are wilted, score = 4 and whole plant is wilted and death, score = 5. Screening was done in periods of 2 months and wilting symptoms were recorded at the

end of this period. Severe stem canker was also scored (Table 1).

### Quantification of bacteria

Two different selective media, SCM-fast (improvement of SCM media) (Koenraad et al. 2009) and D<sub>2</sub>ANX (Chun 1982) were used to quantify bacteria accurately. Accessions representing three different resistance group were used for quantification of bacteria with three different methods. Although screening for resistance was done with five plants, selected accessions for bacteria quantification was done by three plants (out of five plants) and three stem parts of each plant; at inoculation, below the inoculation and above the inoculation point. The stem parts were stored at –80 °C. Extraction from this material was done using PBS buffer (three times the weight of the stem part). For the selective media, 100 µl from 10<sup>4</sup>, 10<sup>5</sup>, 10<sup>6</sup>-fold dilutions were plated in three fold and remaining parts of original extraction from each plant belonging to each accession was mixed regardless to plant part then this extraction was used for TaqMan assay with replication units. Each plate was counted 5 and 7 days after plating (Table 2).

### Colony PCR

In case of doubt, colonies were screened with a colony-PCR method. A colony was picked and suspended and thoroughly mixed in 50 µl MQ. 5 µl of this suspension was diluted with 45 µl 5 mM NaOH solution. This suspension was used as template in the PCR reaction. Primers were chosen based on pCM1, CMM3-4, (Santos et al. 1997) and pCM2, P5-6, (Dreier et al. 1995) and the reaction was done in 50 µl total reaction volume [5 µl PCR buffer solution (10×)], 0.2 µl Taq polymerase (5 U), 1 µl 5 mM Deoxynucleoside triphosphates (dNTPs), 2.5 µl (25 pmol) primer 1 and primer 2.5 µl of DNA template and 33.8 µl dH<sub>2</sub>O PCR conditions were 35 cycles of 94 °C, 30 s; 60 °C, 30 s; 72 °C, 45 s. 15 µl of PCR product and 5 µl loading buffer were separated on 1.5 % agarose TAE gel, and visualized with ethidium bromide.

### TaqMan

DNA extraction was done using the QuickPick SML Plant DNA purification kit provided by Bio-Nobile in

**Table 2** Comparison of bacterial concentration with three different methods: dilution plating on the selective media SCM-fast and D<sub>2</sub>ANX and TaqMan-PCR

Accessions	Plant part	Bacterial titer (cfu/g)		
		SCM-fast	D <sub>2</sub> ANX	TaqMan (average)
<i>S. lycopersicum</i> cv. MM	Upper	$4.00 \times 10^{10}$	$4.60 \times 10^{10}$	$7.2 \times 10^{10} \pm 2.64E+00a$
	Inoculation	$4.22 \times 10^{11} \pm 4.19E+00a$	$5.57 \times 10^{11} \pm 4.83E+00a$	
	Lower	$5.34 \times 10^{11}$	$8.47 \times 10^{11}$	
<i>S. peruvianum</i> LA2157	Upper	$1.83 \times 10^7$	$3.30 \times 10^7$	$5.21 \times 10^8 \pm 7.24E+00bc$
	Inoculation	$3.08 \times 10^8 \pm 6.10E+00bc$	$3.87 \times 10^8 \pm 4.75E+00c$	
	Lower	$5.33 \times 10^8$	$5.90 \times 10^8$	
<i>S. habrochaites</i> LA 407	Upper	$1.32 \times 10^9$	$3.10 \times 10^9$	$1.41 \times 10^9 \pm 4.11E+00bc$
	Inoculation	$2.81 \times 10^9 \pm 1.82E+00b$	$3.39 \times 10^9 \pm 1.57E+00b$	
	Lower	$8.63 \times 10^8$	$1.48 \times 10^9$	
<i>S. lycopersicum</i> cv. IRAT L3	Upper	$9.33 \times 10^8$	$1.32 \times 10^9$	$1.51 \times 10^9 \pm 1.75E+00abc$
	Inoculation	$5.53 \times 10^8 \pm 1.43E+00bc$	$1.26 \times 10^9 \pm 1.20E+00bc$	
	Lower	$1.10 \times 10^9$	$1.77 \times 10^9$	
<i>S. pimpinellifolium</i> GI 1554	Upper	$4.20 \times 10^8$	$1.34 \times 10^9$	$1.79 \times 10^9 \pm 2.45E+00abc$
	Inoculation	$5.74 \times 10^8 \pm 1.34E+00bc$	$1.59 \times 10^9 \pm 1.10E+00bc$	
	Lower	$3.21 \times 10^8$	$1.37 \times 10^9$	
<i>S. peruvianum</i> PI 127829	Upper	$1.20 \times 10^8$	$1.39 \times 10^8$	$1.50 \times 10^8 \pm 6.92E+00c$
	Inoculation	$1.05 \times 10^8 \pm 1.07E+00bc$	$1.39 \times 10^8 \pm 1.05E+00c$	
	Lower	$1.12 \times 10^8$	$1.28 \times 10^8$	
<i>S. parviflorum</i> LA 735	Upper	$4.99 \times 10^8$	$7.80 \times 10^8$	$3.19 \times 10^9 \pm 3.59E+00abc$
	Inoculation	$7.70 \times 10^8 \pm 1.38E+00bc$	$1.08 \times 10^9 \pm 1.29E+00bc$	
	Lower	$9.41 \times 10^8$	$1.28 \times 10^9$	
<i>S. parviflorum</i> LA 2072	Upper	$9.13 \times 10^8$	$1.01 \times 10^9$	$3.79 \times 10^9 \pm 3.20E+00ab$
	Inoculation	$6.95 \times 10^8 \pm 2.23E+00bc$	$7.75 \times 10^8 \pm 2.21E+00bc$	
	Lower	$2.03 \times 10^8$	$2.27 \times 10^8$	
<i>S. peruvianum</i> LA 385	Upper	$2.07 \times 10^8$	$2.70 \times 10^8$	$2.46 \times 10^8 \pm 6.33E+00bc$
	Inoculation	$7.47 \times 10^7 \pm 2.07E+00c$	$1.44 \times 10^8 \pm 1.50E+00c$	
	Lower	$5.03 \times 10^7$	$1.27 \times 10^8$	

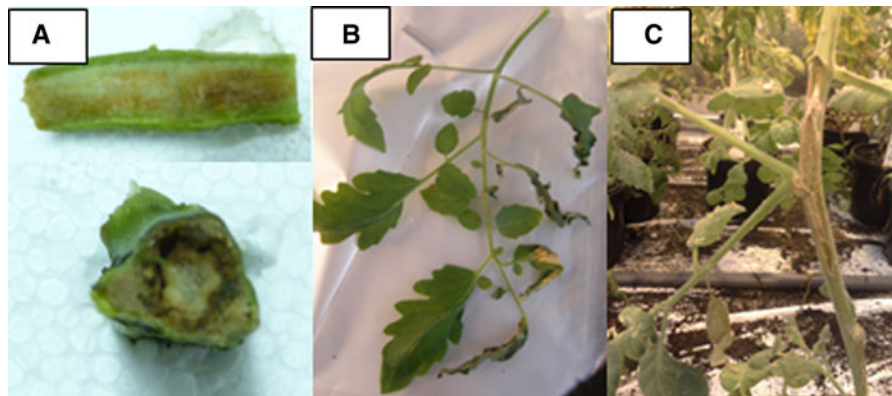
Upper is 30 cm above and lower is 10 cm below the inoculation point. Different letter indicates significant differences between accessions

combination with a Kingfisher processor and followed by a purification step on a PVPP column. The 25  $\mu$ l reaction includes 10  $\mu$ l DNA template, 12.5  $\mu$ l 10 $\times$  Takara mix, 0.5  $\mu$ l Rox and 2  $\mu$ l mix of 4  $\mu$ M Forward primer (GGG GCC GAA GGT GCT GGTG), 4  $\mu$ M Reverse primer (CGT CGC CCG CCC GCTG) and 1  $\mu$ M TaqMan probe with some modification (6-FAM/TGG TCG TCC/ZEN/TCG GCG CC/IABkFQ) (Berendsen 2011). TaqMan probe is based on a chromosomal region of the Cmm sequence. The real-time PCR temperature regime was as follows: 95  $^{\circ}$ C for 30 s followed by 50 cycles of 95  $^{\circ}$ C for 3 s and 60  $^{\circ}$ C for 35 s using Bio-Rad CFX thermocycler.

To obtain a standard curve, three independent replication of tenfold serial dilutions of bacteria was used as template and water control was included as negative control. A plant suspension with Cmm was prepared and diluted to determine the detection level in planta. DNA extractions were done as described above. The standard curve is shown in Fig. 3.

Internal amplification control (IAC) for TaqMan assay

Inhibition of TaqMan (false negative) was checked using *E. coli* O157:H7 strain B6-914 gfp-91 (Peralta



**Fig. 1** Typical symptoms of bacterial disease: stem discoloration (a), unilateral wilting severe (b), and stem canker (c)

et al. 2008) provided by Wageningen University, Plant Research International Biointeractions and Plant Health group. 25  $\mu$ l (containing 5  $\mu$ l sample DNA) or 30  $\mu$ l (containing 10  $\mu$ l sample DNA) PCR solution consisted of 12.5  $\mu$ l 10 $\times$  Takara mix, 0.45  $\mu$ l Rox and 2  $\mu$ l of TaqMan primer's mix (4  $\mu$ M forward, 4  $\mu$ M reverse and 1  $\mu$ M probe), 0.45  $\mu$ l of 5  $\mu$ M GFP forward, 0.45  $\mu$ l of 5  $\mu$ M GFP reverse and 0.3  $\mu$ l of 5  $\mu$ M GFP probe, 0.8 pg GFP DNA and 2  $\mu$ l dH<sub>2</sub>O.

#### Statistical analysis

To determine correlation of three different methods for quantification of Cmm populations, data of each accession was averaged for each method and transformed to a log<sub>10</sub> base mode. After data transformation, data of each method was plotted against another method to see the distribution of data then correlation between methods was done (with SPSS). To check differences between accessions, Anova test using Minitab 16.0 program was used. Different stem parts of each accession were used as replication units for each method to compare means under 95 % confidence level.

## Results

#### Screening of wild tomato species for Cmm resistance

The first symptoms of Cmm were observed as wilted leaves on one side (unilateral) 18 days after inoculation. The appearance of stem canker was variable and accession related. Usually, stem canker occurred at

late stage of wilting but sometimes stem canker appeared before wilting (Fig. 1).

We grouped the screened accessions in three different categories based on wilting score and stem canker severity.

Group 1: accessions with maximum score 1 and 2 (Fig. 2a).

Group 2: accessions with a wilting score 2 with severe stem canker symptoms (Fig. 2b).

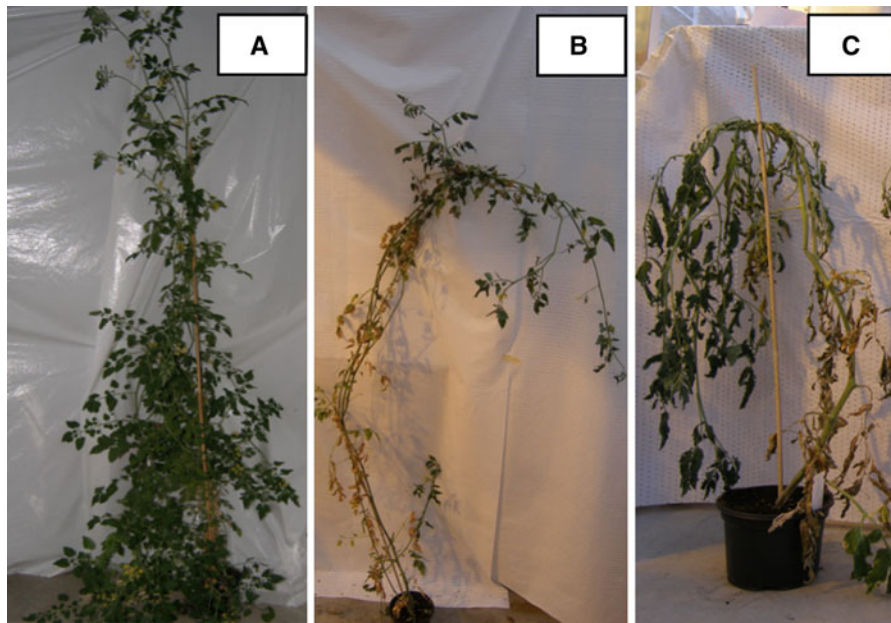
Group 3: a high level of wilting; score 4 and 5 (Fig. 2c).

#### Detection and quantification of Cmm on selective media

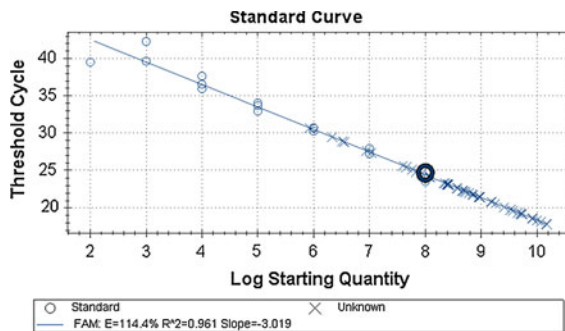
On the semi-selective media D<sub>2</sub>ANX the colonies have after 5 days a yellow, mucoid and convex structure. On the semi-selective media SCM-fast, colonies were visible after 9 days with a grey, mucoid, irregularly morphology and with internal black flecks. On D<sub>2</sub>ANX medium dark yellow and slightly light yellow colored colonies were formed. To confirm the identity of typical colonies, they were tested with a conventional PCR. Based on the sequence of the plasmids of Cmm amplification of two genes involved in virulence were expected, one of 645 bp, primers Cmm 3–4, and one of 614 bp, primers P5–P6. Some colonies gave only one fragment instead of the expected two.

#### Detection and quantification of Cmm by TaqMan PCR

The relation between presence of Cmm bacteria varying from 10<sup>2</sup> to 10<sup>8</sup> cfu/ml and Ct values



**Fig. 2** The effect of bacterial canker on tomato: tolerant-no damage (a), moderately tolerant—middle damage (b) and susceptible—dead plant (c)



**Fig. 3** Detection and quantification of Cmm by TaqMan-PCR. A tenfold serial dilution was tested in three replicates and the Ct values are plotted against the log of the bacterial concentration. A semi-log regression line plot of the Ct value is shown versus the log of the bacterial densities

(threshold cycle value) is shown in Fig. 3. A standard curve obtained between bacteria concentration and Ct value gave a correlation coefficient of 0.961. It was possible to detect bacteria till  $10^3$  cfu/ml, below this threshold bacteria are still detectable but less reliable. Therefore the detection limit was set on  $10^3$  cfu/ml. Detection level for plant extract containing serial dilution of bacterial suspension was also  $10^3$  cfu/ml (data not shown). The CT values of the bacterial

internal control GFP were the same in reactions containing 0, 5 and 10  $\mu$ l DNA sample DNA. In case of standard dilutions and samples, same CT values were obtained with or without GFP DNA amplification (data is not shown). There was no IAC co-amplification influence on standard dilution and samples PCR.

#### Bacterial concentration of screened tomato species

Cmm quantification was done with some of the accessions, representing the three different groups; tolerant, moderate and susceptible. Quantification was done by two selective mediums and TaqMan PCR. The results are given in Table 2 where the concentration Cmm in one gram plant material is given based on the three different detection methods. There was a good correlation in results between two selective mediums (0.99), between D<sub>2</sub>ANX medium and TaqMan (0.92), and between SCM-fast medium and TaqMan assay (0.92). Bacteria concentrations in the inoculated wild accessions ranged from  $10^7$  to  $10^{11}$  cfu/ml. The susceptible control accession *S. lycopersicum* cv. Moneymaker had the highest titer of bacteria and also had the highest disease score.

## Discussion

Screening of 24 wild species including accessions with a known level of tolerance identified new tolerant sources and confirmed others. Wild species of tomato have been used to increase the gene pool of tomato; this is needed especially for the introduction of resistances to diseases and pests. In our study we have screened for tolerance to *Clavibacter michiganensis* subsp. *michiganensis* (Cmm). The tolerance of *S. pimpinellifolium* GI.1554, *S. parviflorum* LA 735 and *S. parviflorum* LA 2072 accessions has not been reported before. We have confirmed a high tolerance in *S. peruvianum* LA 2157, *S. peruvianum* PI 127829 and *S. peruvianum* LA 385 (Lindhout and Purimahua 1987), and a moderate tolerance in *S. habrochaites* LA 407 (Francis et al. 2001) and *S. lycopersicum* cv. IRAT L3 (Laterrot et al. 1978). The accession *S. peruvianum* LA 2157 (van Heusden et al. 1999) was the most tolerant in our screening as it was reported before. All *S. cheesmanii* accessions were very susceptible. Morphological differences may be involved in resistance to Cmm (Coaker et al. 2002). *Solanum cheesmanii* accessions all have a typical, succulent and easy breaking, stem. This difference in stem morphology might be the reason for the extreme susceptibility.

Dilution plating on selective media was successfully used to detect population densities in the different accessions. Different Cmm strains exhibit variation in growth characteristics, including colony structure and morphology (Eichenlaub et al. 2006). Because of that, at least two different mediums are advised to quantify Cmm in plant material (Anonymous 2008; Hadas et al. 2005). In our study, the densities of cfu of Cmm on the two semi-selective media, D<sub>2</sub>ANX and SCM-fast, were measured and the numbers were generally higher on D<sub>2</sub>ANX. We observed some colonies of saprophytes on media D<sub>2</sub>ANX but not on SCM-fast which indicates a better selectiveness of the SCM-fast media. Some colonies were screened with the Colony-PCR method using genes involved in pathogenicity and it was confirmed that they were Cmm containing virulence genes. Few colonies showed an aberrant colony-morphology and did not amplify with one of the primer combinations indicating that one plasmid is missing. It has been reported that the presence of plasmids of Cmm is not stable (Gartemann et al. 2003) and those lost plasmid can be cured Cmm during experiment. Since Cmm

with missing plasmid were small proportion of population, we ignore their effect on disease score. Also repeated experiment on most resistant accessions, *S. pimpinellifolium* GI.1554 and *S. peruvianum* LA 2157, with same strains in another experiment resulted in same observation.

We developed an indirect TaqMan real time PCR to identify and quantify Cmm *in planta*. Dilution plating on selective media to detect Cmm is the advised method by the International Seed Federation and this method has been used for decades to identify and quantify Cmm (Fatmi and Schaad 1988). Although this is a reliable method it is very laborious and it takes 5–7 days to grow bacteria to countable colonies. In addition, confirmation of the nature of colonies is needed by other methods. We used successfully an internal amplification control (IAC) to excluded false-negative results which did not affect the sensitivity of our TaqMan assay (results not shown). The detection level in our study was determined at a level of 10<sup>3</sup> cfu/ml. The sensitivity was sufficient to detect the relatively high densities present in stems. A high, significant correlation between Ct values in the TaqMan assay and the concentrations based on the dilution plating on selective media was found. In this study, we are reporting new Cmm tolerance sources in crossable wild relatives of tomato. Although these sources have high tolerance levels, they still contain substantial numbers of bacteria. We didn't find a resistance source that was completely free of bacteria. In general, there is correlation of bacteria concentration and resistance level. Susceptible accessions had 10–1,000-fold more bacteria than the tolerant sources, but the bacterial concentration among tolerant accessions varies. This might be due to different resistance mechanisms and that the fact that a lack of symptom expression is not only based on reduction of bacteria. (Coaker et al. 2004). To determine the systemic translocation of bacteria in the plant, we have checked three different parts of the plants of each accession; the cotyledon, the inoculation point and stem above the inoculation point. Bacteria spread both upwards and downwards in the stem, this is in contradiction with previous reports where bacteria distribution was irregular and unpredictable (Van Steekelenburg 1985). In our opinion bacteria spread irregularly and unpredictable only at the beginning of infection, but later it invades each part of the plant via xylem vascular tissues. Looking at the sequence in which

wilted leaves appear, there is an indication that the bacteria first move upwards and later downwards and into side shoots.

In conclusion, new tolerant sources have been identified. Of one of these sources (*S. pimpinellifolium* GI.1554) we have a genetically well studied recombinant inbred line population. This population will be used for a QTL mapping study and interactions with the described QTLs from *S. peruvianum* LA2157 will be investigated. To be able to do this we are developing nearly isogenic lines. Our aim is to develop the tools to make new cultivars with a high tolerance to Cmm and preferably no transmission via seeds. This will especially be an advantage for growers but seed companies can only sell seeds completely free of Cmm. A TaqMan assay is suitable for quantitation of Cmm in stem inoculated accessions of tomato with a different level of susceptibility.

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## References

- Anonymous (2000) European Union Council directive 2000/29/EC on protective measures against the introduction into the Community of organisms harmful to plants or plant products and against their spread within the Community. Off J Eur Commun L 169(1):33
- Anonymous (2008) ISHI Protocol for the Detection of *Clavibacter michiganensis* subsp. *michiganensis* on Tomato Seeds by Isolation on Media Version 3
- Bach HJ, Jessen I, Schlöter M, Munch JC (2003) A TaqMan-PCR protocol for quantification and differentiation of the phytopathogenic *Clavibacter michiganensis* subspecies. J Microb Methods 52(1):85–91
- Berendsen SMH, Koenraad H, Woudt B, Oosterhof J (2011) The development of a specific Real-Time TaqMan for the detection of *Clavibacter michiganensis* subsp. *michiganensis*. In: APS-IPPC Meeting, Honolulu
- Carlton WM, Braun EJ, Gleason ML (1998) Ingress of *Clavibacter michiganensis* subsp. *michiganensis* into tomato leaves through hydathodes. Phytopathology 88(6):525–529
- Chun WCC (1982) Identification and detection of *Corynebacterium michiganense* in tomato seed using the indirect enzyme-linked immunosorbent assay. MSc thesis, University of Hawaii, Honolulu
- Coaker GL, Meulia T, Kabelka EA, Jones AK, Francis DM (2002) A QTL controlling stem morphology and vascular development in *Lycopersicon esculentum* × *Lycopersicon hirsutum* (Solanaceae) crosses is located on chromosome 2. Am J Bot 89(12):1859–1866
- Coaker GL, Willard B, Kinter M, Stockinger EJ, Francis DM (2004) Proteomic analysis of resistance mediated by Rcm 2.0 and Rcm 5.1, two loci controlling resistance to bacterial canker of tomato. Mol Plant Microbe Interact 17(9):1019–1028
- Dreier J, Bermpohl A, Eichenlaub R (1995) Southern hybridization and PCR for specific detection of phytopathogenic *Clavibacter michiganensis* subsp. *michiganensis*. Phytopathology 85:462–468
- Eichenlaub R, Gartemann KH, Burger A (2006) *Clavibacter michiganensis*, a group of gram-positive phytopathogenic bacteria. In: Plant-associated bacteria, Springer Netherlands
- Elenkov E (1965) Die selektion von tomaten auf resistenz gegen die bakterienwelke. Int Z Landwirt 594–597
- Emmatty DA, John CA (1973) Evaluation of resistance to bacterial canker of H2990, a new tomato variety. Plant Dis Report 57(7):584–586
- Fatmi M, Schaad NW (1988) Semiselective agar medium for isolation of *Clavibacter michiganense* subsp. *michiganense* from tomato seed. Phytopathology 78(1):121–126
- Francis DM, Kabelka E, Bell J, Franchino B, Clair DS (2001) Resistance to bacterial canker in tomato (*Lycopersicon hirsutum* LA407) and its progeny derived from crosses to *L. esculentum*. Plant Dis 85(11):1171–1176
- Gartemann KH, Kirchner O, Engemann J, Grafen I, Eichenlaub R, Burger A (2003) *Clavibacter michiganensis* subsp. *michiganensis*: first steps in the understanding of virulence of a Gram-positive phytopathogenic bacterium. J Biotechnol 106(2–3):179–191
- Hadas R, Kritzman G, Klietman F, Gefen T, Manulis S (2005) Comparison of extraction procedures and determination of the detection threshold for *Clavibacter michiganensis* ssp. *michiganensis* in tomato seeds. Plant Pathol 54(5):643–649
- Hassan AA, Strider DL, Konsler TL (1968) Application of cotyledonary symptoms in screening for resistance of tomato to bacterial canker and host range studied. Phytopathology 58:233–239
- Koenraad H, van Vliet A, Neijndorff N, Woudt B (2009) Improvement of semiselective media for the detection of *Clavibacter michiganensis* subsp. *michiganensis* in seeds of tomato. (Abstr.) Phytopathology 99:S66
- Kuriyama T, Kuniyasu K (1974) Studies on the breeding of resistant tomato by interspecific hybridization. III. On the breeding of a new tomato line resistant to bacterial canker caused by *Corynebacterium michiganense*. Bull Veg Ornament Crops Res Stn Jpn A 1:93–107
- Laterrot H, Brand R, Daunay MC (1978) La résistance à *Corynebacterium michiganense* chez la tomate Étude bibliographique. Ann Amélior Plantes 28:579–591
- Lindhout P, Purimahua C (1987) Resistance against *Corynebacterium michiganense* found in *Lycopersicon peruvianum*. In: Synopses 10th meeting Eucarpia Tomato working group. Pontecagnano, Italy
- Lindhout P, Purimahua C (1989) Investigation on tomato breeding for resistance to *Corynebacterium michiganense*. In: XIIth eucarpia congress, Göttingen
- Luo LX, Walters C, Bolkan H, Liu XL, Li JQ (2008) Quantification of viable cells of *Clavibacter michiganensis* subsp.



- michiganensis* using a DNA binding dye and a real-time PCR assay. *Plant Pathol* 57(2):332–337
- Peralta IE, Spooner DM, Knapp S (2008) Taxonomy of wild tomatoes and their relatives (Solanum sect. Lycopersicon, sect. Juglandifolia, sect. Lycopersicon; Solanaceae). *Syst Bot Monogr* 84:1
- Poysa V (1993) Evaluation of tomato breeding lines resistant to bacterial canker. *Can J Plant Pathol* 15(4):301–304
- Santos MS, Cruz L, Norskov P, Rasmussen OF (1997) A rapid and sensitive detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seeds by polymerase chain reaction. *Seed Sci Technol* 25(3):581–584
- Sotirova V, Achkova ZI (1989) Resistance of tomatoes to *Corynebacterium michiganense* (Smith) Jensen. In: XIIth eucarpia congress, Göttingen
- Sotirova V, Bogatsevska N, Stamova L (1994) Sources of resistance to bacterial diseases in tomato wild species. *Acta Hort* 376:353–360
- Thyr BD (1976) Inheritance of resistance to *Corynebacterium michiganense* in Tomato. *Phytopathology* 66:1116–1119
- van Heusden AW, Koornneef M, Voorrips RE, Bruggemann W, Pet G, Vrielink van Ginkel R, Chen X, Lindhout P (1999) Three QTLs from *Lycopersicon peruvianum* confer a high level of resistance to *Clavibacter michiganensis* ssp. *michiganensis*. *Theor Appl Genet* 99(6):1068–1074
- Van Steekelenburg NAM (1985) Resistance to *Corynebacterium michiganense* in tomato genotypes. *Euphytica* 34: 245–250
- Zhao WJ, Chen HY, Zhu SF, Xia MX, Tan TW (2007) One-step detection of *Clavibacter michiganensis* subsp. *michiganensis* in symptomless tomato seeds using a TaqMan probe. *J Plant Pathol* 89(3):349–351