Bacterial Canker Resistance in Tomato

Yusuf Şen

Thesis committee

Promotor

Prof. Dr R.G.F. Visser

Professor of Plant Breeding

Wageningen University

Co-promotor

Dr A.W. van Heusden

Scientist, Wageningen UR Plant Breeding

Wageningen University and Research Centre

Other members

Prof. Dr M. Koornneef, Wageningen University

Prof. Dr R.A.A. van der Vlugt, Wageningen University

Dr E. Heuvelink, Wageningen University

Dr F. Millenaar, Bayer CropScience Vegetable Seeds, Haelen

This research was conducted under the auspices of the Graduate School of Experimental Plant Sciences.

Bacterial Canker Resistance in Tomato

Yusuf Şen

Thesis

submitted in fulfilment of the requirements for the degree of doctor

at Wageningen University

by the authority of the Rector Magnificus

Prof. Dr M.J. Kropff,

in the presence of the

Thesis Committee appointed by the Academic Board

to be defended in public

on Monday 29 September 2014

at 11 a.m. in the Aula.

Yusuf Şen

Bacterial Canker Resistance in Tomato,
140 pages.

PhD thesis, Wageningen University, Wageningen, NL (2014)
With references, with summaries in English and Dutch

ISBN: 978-94-6257-076-4

Table of Contents

İlim, ilim bilmektir

ilim, kendin bilmektir

Yunus Emre

Chapter 1

General introduction

General introduction

Clavibacter michiganensis subsp. michiganensis (*Cmm*) is an aerobic non-sporulating gram-positive plant pathogenic bacterium. It belongs to the genus Clavibacter and currently this genus is comprised of five subspecies; *C. m. subsp. michiganensis, C. m. subsp. sepedonicus, C. m. subsp. nebraskensis, C. m. subsp. insidiosus and C. m. subsp. tessellarius.*

Clavibacter michiganensis subsp*. michiganensis* (*Cmm*) is the causal agent of bacterial canker in tomato and was for the first time described in 1910 in Michigan, USA [1]. The host range of the pathogen is mainly in *Solanaceae* crops such as tomato, pepper and eggplant. In nature *Cmm* has different virulence levels; hyper virulent, reduced virulent and non-virulent. The genome of *Cmm* strain NCPPB382 has been sequenced [2]. *Cmm* harbors high numbers of transporters and transcriptional regulators and is therefore very similar to soil bacteria, indicating that *Cmm* is a recently evolved pathogen that evolved from plant-associated Microbacteriaceae [2].

Bacterial canker caused by *Cmm* is considered the most important bacterial disease in tomato and yield losses can be severe. *Cmm* is a quarantine organism in the European Union and in many other countries [3]. During early stages of disease development, unilateral wilting of leaflets and leaves is common. Cankers, which gave their name to the disease, develop on stems and petioles in later stages of infection. Symptoms on tomato fruits often are seen as small, tan lesions surrounded by white halos which are called bird's-eye spots. Foliar symptoms are small, white, blister like spots on the leaves. As disease progresses yellow-to-brown regions of marginal necrosis referred to as "firing" symptoms develop on leaflets of diseased plants [4]. Transmission occurs via contaminated seeds, but infection of *Cmm* also occurs through stomata, roots, damaged tissue, and other natural openings. The main source of the spreading of *Cmm* in the field and in greenhouses is cultural practice. After infection *Cmm* invades the xylem vessel, which is followed by a systemic infection of the host. The infection cycle of *Cmm* is depicted in Figure 1.

There are several outbreak reports of *Cmm* infestations from all over the world [5]. Yield losses due to bacterial canker vary per year, location, cultivar and time of infection [6]. Sensitive and reliable detection is crucial in order to properly identify the pathogen and to prevent its transmission. In general, four methods are in use: serological methods, genetic methods, bioassays and dilution plating. Control of disease by growers is carried out mainly by chemicals but use of clean materials and clean cultural practices are also advised as effective tool to prevent pathogen spread. However, the most effective and environmental friendly way to prevent and control the disease is to use resistant varieties. Unfortunately, commercially no cultivars are known with an effective resistance to *Cmm*.

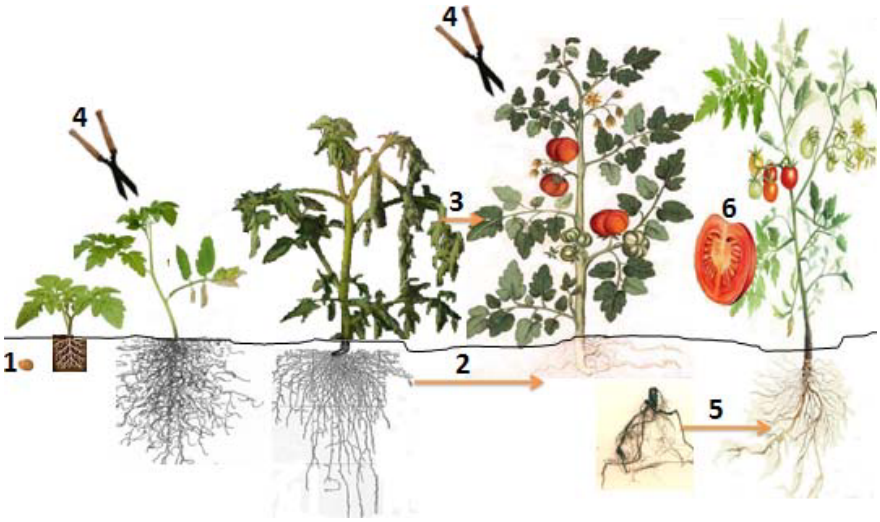


Figure 1. Infection cycle of *Cmm* in tomato. Cycle starts with infected seeds (1), continues with spreading towards roots (2), leaves (3) and seeds (6). Cultural practices (4) and debris contamination (5) can also be reasons that the contamination is again present in the next growth cycle.

Outline of this Thesis

The studies presented in this thesis aim to describe tools and materials for *Cmm* research. In this way, not only high quality advanced breeding material can be produced which might lead to *Cmm* resistant cultivars, but also effective methods to monitor any disease outbreak and/or development of the disease. We have

developed different approaches and advanced materials for further research. We also identified genomic regions associated with resistance and hypothesized about possible plant resistance mechanisms and pathogen virulence pathways.

Chapter 2 is a review aimed at describing the available knowledge about *Cmm* including biology and epidemiology of the pathogen, disease management, *Cmm* detection, plant genetic resources for resistance, genetic analysis of those sources, plant resistance mechanisms, bacterial movement in the plant and plant-microbe interactions. *Cmm* is a long term problem in tomato production areas and scientifically it did not get much attention. Due to a lack of knowledge the disease management is not sufficient. No resistance genes, preventing bacterial multiplication, are available for breeders.

Chapter 3 describes the use of real time TaqMan PCR as plant phenotyping technique by which detection and quantification of *Cmm* is reliable. With this technique, the resistance level of 24 wild tomato species was evaluated based on their response to one aggressive *Cmm* strain. The relation of bacterial concentration and the level of wilting was determined in the accessions. Based on wilting and bacterial concentration the resistance levels of previously known sources were confirmed and new sources for *Cmm* resistance were found.

Chapter 4 describes the genetic analysis of one of the resistance sources (*S. pimpinellifolium*) identified in Chapter 3. The genetic analysis was performed in a Recombinant Inbred Line population using three different parameters: bacterial concentration, wilting and stem discoloration. A high density genetic map was constructed using the Infinium SNP array genotyping technique. This population was screened under three different conditions. Due to multi-environmental screening and multi-trait phenotyping, the power of multi-trait multi-environment QTL approach compared to a single QTL approach was discussed.

Chapter 5 describes the fine mapping of previously known QTLs by use of old stock genetic material and two different SNP genotyping technologies; High Resolution

Melting (HRM) and KBioscience Allele Specific PCR (KASP). Development of nearly isogenic lines containing those known QTLs by overcoming interspecific genetic barriers and marker assisted background selection is also described.

Chapter 6 describes a characterization of 108 new Turkish *Cmm* strains. Differentiation of the strains was done using serological, molecular, hypersensitive response and pathogenicity tests. The *Cmm* strains, which were collected over a period of 20 years in different times of the year and in different fields in Turkey, were analyzed by a Multilocus Sequence Typing (MLST) approach using different housekeeping genes and virulence related genes. Clonal complex analyses and split network analyses were done for this collection and a phylogenetic tree was constructed. By adding reduced virulence strains, strains from other collections and strains representing different *Cm* subspecies, the relation between this collection and other collections was made and the relation between hyper virulent, less virulent and other subspecies was determined.

Chapter 7 summarizes the main results obtained in this thesis, and gives concluding remarks and future perspectives.

Chapter 2

Bacterial canker in tomato: status of knowledge on resistance, detection, management and interaction

Submitted for publication

Bacterial canker in tomato: status of knowledge on resistance, detection, management and interaction

Yusuf Sen[1,2], Jan van der Wolf [3] , Richard G.F. Visser[1], Sjaak van Heusden[1,4]

(1) Wageningen UR Plant Breeding, Wageningen University and Research Centre, PO Box 386, 6700 AJ Wageningen, The Netherlands (2) Graduate School Experimental Plant Sciences, Wageningen Campus, 6807PB Wageningen, The Netherlands. (3) Plant Research International Biointeractions and Plant Health, Droevendaalsesteeg 1, 6708 PB, Wageningen, The Netherlands. (4) Corresponding author, Email: sjaak.vanheusden@wur.nl.

Abstract

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*) is an aerobic non-sporulating gram-positive plant pathogenic bacterium and the causal agent of bacterial canker in tomato. It is considered the most harmful bacterial pathogen of tomato and is under quarantine regulation in many countries. Lack of knowledge on the complex behavior of *Cmm* as plant pathogen, the lack of tools to control the disease and the lack of resistant genotypes hampered the introduction of successful management tools. Resistance mechanisms of reported plant/species sources and the specific host-pathogen interaction system have still remained elusive. In this review, we discuss the biology of *Cmm* as plant pathogen, management tools including the use of methods for seed and plant testing and extensively the perspectives for resistance breeding.

Keywords: Clavibacter, tomato, detection, resistance, interaction

Clavibacter michiganensis

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*) is an aerobic non-sporulating gram-positive plant pathogenic bacterium and the causal agent of bacterial canker in tomato. It is a quarantine organism in the European Union and in many other countries [3]. *Cmm* has been described as a phloem parasite. Later, it was shown that in fact *Cmm* is a xylem invading bacterium [7]. Being a mesophilic bacterium *Cmm* can successfully grow from 20 to 30 ºC. The optimum growth temperature is 25 ºC but *Cmm* can survive up to 50 ºC. It is possible to grow *Cmm* on artificial medium and it takes 3 to 7 days before colonies become visible on selective agar plates. The

optimum pH is between 7 and 8 but at pH 5 in the xylem *Cmm* still grows [8]. Strains of *Cmm* can largely vary in their virulence [9]. Molecular typing of *Cmm* strains based on genomic fingerprinting (rep-PCR) analysis revealed four haplotypes [10-12]. A combination of rep-PCR and multilocus sequence typing (MLST) analysis, identified seven groups of *Cmm* strains [13]. The genome of *Cmm* strain NCPPB382 has been sequenced. The circular chromosome consists of 3,298 Mb and has a high GC content (72.6%). In total 2,984 coding DNA sequences were found, of which 2,029 could be annotated. About 20 *Cmm* specific regions with a low GC content were found. The largest one is the chp/tomA region. The low GC content regions are thought to be of foreign origin introduced via horizontal gene transfer [2].

Bacterial Canker

Cmm infestation has been already for a long time a problem worldwide (Strider, 1969). Bacterial wilt caused by *Cmm* is certainly the most important bacterial disease in tomato [14]. During the early stages of disease development, unilateral wilting of leaflets and leaves is common (Fig. 1A). Cankers, from which the disease got its name, develop on stems and petioles in later stages of pathogenesis (Fig. 1B). On infected tomato fruits incidentally characteristic birds-eye spots develop. These consist of small, dark lesions surrounded by white halos. Foliar symptoms occasionally include small, white, blister like spots. More commonly, yellow-to-brown regions of marginal necrosis, sometimes referred to as “firing” symptoms, develop on leaflets of diseased plants [4]. At the very end stage of infection whole plant death is observed (Fig. 1C). Stem discoloration is a symptom of this disease [15].

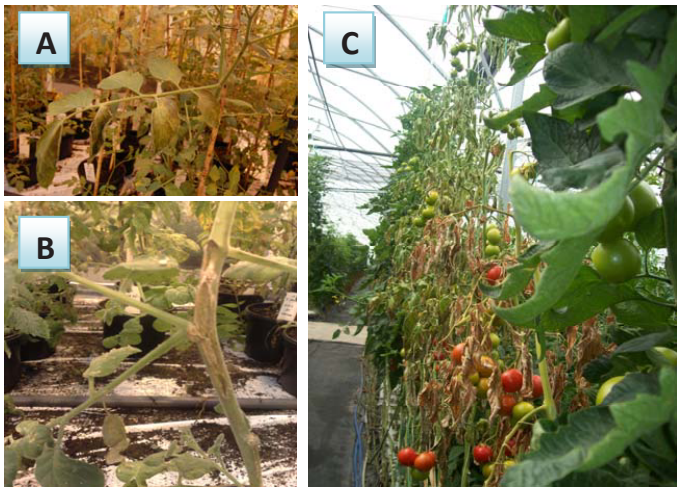


Figure 1: Common symptoms of bacterial canker: unilateral wilting (A) , stem canker (B), dead plants (C).

Life cycle

Infected seed is one of the infection sources of disease outbreaks [12]. In addition, seed is the main long distance vector of the pathogen. The transmission of *Cmm* from seed to seedling can vary from 0.25% to 85% [5]. Densities as low as five cells per seed can result in infected seedlings [16]. Even a transmission rate of 0.01% can cause serious epidemic under favorable conditions [17]. The spread of *Cmm* within an infected crop mainly occurs by cultural practices such as pruning, clipping, contact infections, splash dispersal and via nutrient solutions [4, 18, 19]. Secondary infections of *Cmm* occur through stomata, hydathodes, roots and damaged tissues including damaged trichomes [20]. After infection, *Cmm* invades xylem vessels, which is followed by a systemic infection of the host. The infection cycle of *Cmm* has been described [8]. Infection of tomato plants at a later stage of growth can result in symptomless plants but the seeds might still be contaminated. Yield losses due to bacterial canker vary with year, location, cultivar and time of infection. Bacterial canker can drastically reduce yields. In Canada (Ontario) it accounted for yield losses up to 84% in commercial fields. In artificially infected crops, it varied from 46% to 93% [6]. The economic losses can be high; in Michigan, USA it caused on average an estimated annual loss of 300.000$ [12]. *Cmm* can survive in the soil, in association with plant debris, for about two years [21].

Disease management

Disease management strategies can be grouped in two categories; prevention and control. Disease management by means of control against *Cmm* can involve chemical and biological treatments. Antimicrobial compounds such as copper sulphate, copper hydroxide, copper hydroxide/mancozeb, streptomycin or streptomycin/copper hydroxide are known to reduce the spread and disease incidence of *Cmm*. Currently no chemicals can fully control *Cmm* [3, 22]. The use of copper compounds can result in phytotoxic effects [23]. Some organic antimicrobial substances can reduce bacterial spread, examples are lysozyme, fragarin [24], endolysins of bacteriophages [25], and plant essential oils [26].

In general, chemicals can also be used to activate the plant defense system in a constitutive way, resulting in general barriers against invasion of the pathogen, in production of compounds involved in the defense mechanism, or via priming. With priming the defense mechanism is induced after recognition of the pathogen. Chemicals that have been described for resistance induction are salicylic acid, jasmonic acid and specific volatiles such as nitric oxide and ethylene, DL-β-aminobutric acid (BABA) [27], salicylic acid (SA), potassium salts, 2,6-dichloroisonicotinic acid (INA), acibenzolar-S-methyl [28].

Attempts have been made to control *Cmm* with biocontrol agents. A treatment with *B. subtilis* [29] or a pre-inoculation with avirulent *Cmm* [30] reduces bacterial spread. The mechanism by which *Cmm* is controlled is not known. Growing practices such as lowering the pH of the growth solution can limit the growth of bacteria [31].

Although the rate of control by means of chemical treatments and biological treatments is reported to be significant, this rate was not found to be economically sufficient to be applied and consequently these components are not widely used.

Beside control of disease by means of chemical or biological methods, prevention can be done by means of hygienic measures such as using clean propagation materials, clean materials, clean water and clean humans. The organization, Good Seed and Plant Practices (GSPP), founded in the Netherlands and France aims to prevent tomato seed and plant lots from being infected by *Clavibacter michiganensis subsp. michiganensis* (*Cmm*) (http://www.gspp.eu/). The use of GSPP and the application of control agents is expected to strongly support production of pathogen-free seed and planting material.

Prevention and control measures are supportive in disease management, however the use of resistant cultivars would be the most effective and environmentally friendly method. For commercial seed trade there is zero tolerance for the presence of *Cmm* but no commercial cultivars harboring substantial levels of resistance to *Cmm* are on the market.

Detection

Cmm is regulated as a harmful organism by the European Community (Annex II A section II of Directive 2000/29/EC as amended). The availability of sensitive and reliable (specific and robust), fast and cheap detection methods are indispensable in disease management strategies for this pathogen. Detection is also important in plant material in track and trace studies in case of outbreaks. For seed, the International Seed Federation requires that in 10,000 seeds no *Cmm* can be found using two selective media for *Cmm* in parallel [32].

Different *Cmm* detection methods are known: serological methods, genetic methods, bioassays and dilution plating (Fig. 2). A generally accepted *Cmm* detection method is based on dilution plating on semi-selective media, SCM and D_2ANX, and confirmation of suspected colonies by a bio-assay [33], the weakness and strength of each detection method will be discussed below.

Serological methods that are described for *Cmm* detection are ELISA, immunofluorescence (IF), immunofluorescence colony staining (IFC) and immunomagnetic bead separation (IMS). Dead and viable cells cannot be distinguished and cross reaction with other saprophytes might occur [34]. The

specificity and the detection level of ELISA is dependent on the quality of the antibodies and type of antibodies (monoclonal or polyclonal). A monoclonal antibody against *Cmm* was produced that is able to detect 99% of the *Cmm* strains [35]. But cross reaction of polyclonal antibodies with some other saprophytes has been observed [36]. The detection level of the ELISA varies between 10^3 cfu/ml and 10^4 cfu/ml [37]. ELISA is used routinely to detect *Cmm* [38] and ELISA kits are commercially available. In immunofluorescence (IF) individual bacterial cells, bind to a microscope glass by heat or alcohol fixation, and are stained with antibodies conjugated with a fluorophore that bind to outer cell wall compounds. The stained cells can be visualized with epifluorescence microscopy. The detection level is 10^3 cfu/ml in naturally contaminated seeds [39]. In routine testing programs, to confirm the presence of *Cmm* in samples positive in immunofluorescence techniques (IF), samples are plated on selective media. Immunofluorescence colony staining (IFC) identifies immunostained target colonies. The assay takes 3-5 days to complete, because it is based on agar mixed plating of samples till small, disk-formed colonies are formed, which are subsequently stained with fluorophore labeled antibodies. Consequently, in IFC only living bacteria are detected. Relative large amounts of antibodies are used which makes the technique expensive. Down to 10 cfu/ml can be detected [40] and it is therefore ten times more sensitive than the IF method [41]. Immunomagnetic bead separation (IMS) is based on the use of immunomagnetic beads coated with specific antibodies to capture target bacterial cells allowing removal of non-targets prior to plating on a non-selective medium. The detection threshold is 10 cfu/ml in a heterogeneous seed mixture [42]. IMS-plating is sensitive compared to dilution plating on semi selective media, immunofluorescence (IF), ELISA and PCR techniques [43]. IMS-plating can also be used in combination with PCR (IMS-PCR).

Genomic methods are based on the detection of specific nucleic acid sequences, e.g. by DNA amplification via polymerase chain reaction (PCR). The *Cmm* sequence is available and the pathogenic region of *Cmm* is known. DNA primers based on the sequence of the pathogenic region, celA and pat-1, can distinguish *Cmm* from other *C. michiganensis* subspecies and make it possible to distinguish virulent and avirulent *Cmm* strains. The detection level of the PCR method in plant homogenates is 10^2 cfu/ml [37], a disadvantage of this technique is that it doesn't distinguish dead from viable cells. Bio-PCR (bacteria cultured on agar media prior to PCR) is a sensitive technique that predominantly detects viable cells. One infected seed per 10,000 seeds can be found with the Bio-PCR technique [44]. PCR and Bio-PCR do not allow to quantify *Cmm* but only show whether *Cmm* is present or not. For quantification, Real Time PCR (RT-PCR) can be used but this technique can result in wrong conclusions due to dead cells or PCR inhibitors [45]. TaqMan RT-PCR has been used to quantify and differentiate *C. michiganensis* subspecies in buffer suspensions [46]

but the sensitivity level was not mentioned. In our study [15] we were able to detect bacteria down to 10^2 cfu/ml. TaqMan RT-PCR in combination with ethidium monoazide (EMA) could differentiate dead and viable cells with a detection level of 10^3 viable cells/ml [47].

Dilution plating on selective media allows quantification, isolation and full identification of viable cells of the target pathogen. The technique is relatively time consuming and laborious; it takes 5-14 days to obtain results. The efficiency is dependent on the microbial background as growth of *Cmm* colonies can be inhibited by other microorganisms. *Cmm* shows a great variability in growth characteristics on the different selective media and therefore the use of more than one medium in parallel is recommended. Selective media are mSCM, SCM, SCM-fast, D_2ANX, CNS and non-selective are YDS and NBY [48-50]. With selective medium (SCM medium) the presence of one infected seed (with 50 bacteria) in 10,000 uninfected seeds can be found [48]. Inclusivity (target) and exclusivity (non-target) from tomato seed was checked with SCM and D_2ANX and both media showed an inclusivity and exclusivity between 77 and 87%. The detection level was 1 to 10 cfu/ml [39]. Recently a new medium, BCT, which showed a better recovery in the presence of a high saprophytic background has been introduced [51]. Although media plating is very sensitive false negative results can be caused by low levels of infection [39] and by high microbial backgrounds (van der Wolf, unpublished results) .

Bioassays are based on inoculation of susceptible tomato plants with pure bacterial culture and observing whether typical *Cmm* symptoms occur in these plants. Bioassay tests are used to distinguish *Cmm* lookalikes from *Cmm* and confirm the level of virulence of *Cmm* strains [52, 53]. The colony morphology and color of *Cmm* and *Cmm* lookalikes can be very similar [44]. It is further known that *Cmm* strains can largely vary in the level of virulence level; hyper virulent, hypo virulent and avirulent *Cmm* strains exist [9]. Bioassays on seedlings require at least 21 days [33, 34] before *Cmm* symptoms can be observed.

In summary, each method has its own advantages and disadvantages. Serological methods are relatively fast and cheap but have the risk of cross reaction (aspecificity) with other organisms (false positives) and the sensitivity is limited which can result in false negative results. Molecular techniques can also have problems with specificity (false positives) or with PCR inhibitors (false negatives). In plating techniques, *Cmm* can be overgrown by non-target pathogens or overestimated because of lookalikes. The identity of colonies needs to be re-examined by other methods. For the bioassays, pure cultures are required and procedures take weeks to complete. Depending on the sample (seed or plant), purpose (detection or quantification), time (fast or slow) different methods or a combination of methods can be used for detection and quantification of *Cmm*.

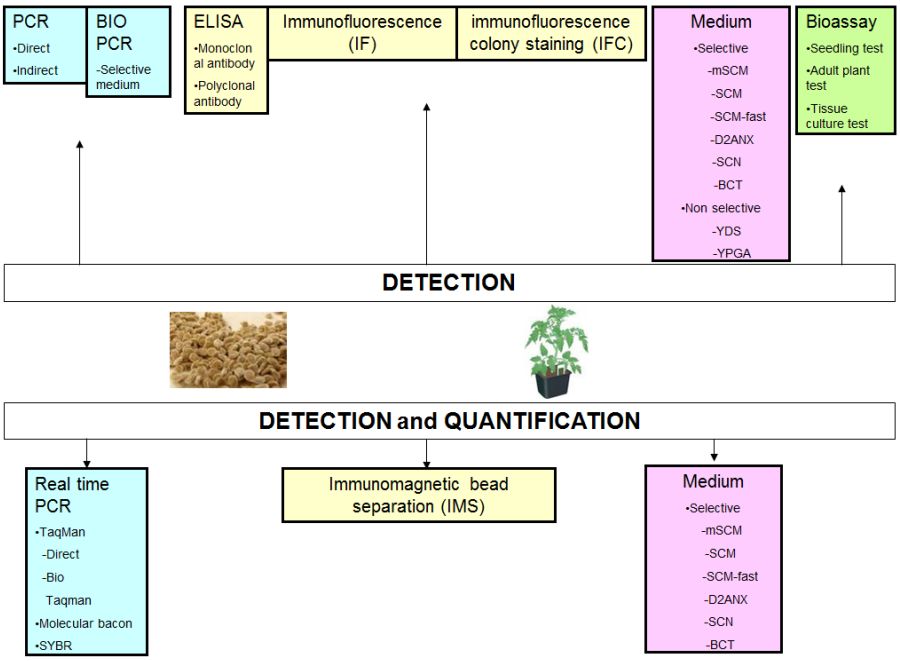


Figure 2: General scheme of detection and quantification methods of *Cmm* in tomato plants and seeds.

Resistance sources

Resistance has been found in several *Solanum* species. Resistance was first reported in *S. pimpinellifolium* (L.) Mill (Anonymous, 1934 cited in [54]). Later, in *S. habrochaites* [55-58], in *S. pimpinellifolium* [15, 54, 56, 57, 59], in *S. lycopersicum* derived lines [6, 60-67], in *S. arcanum* and *S. peruvianum* [57, 68] and in *S. chilense* [56, 57]. Resistant *S. lycopersicum* lines such as the Bulgaria 12 variety with small fruits [60], Heinz 2990 [62] and Okitsu sozai 1-20 [64] originating from *S. pimpinellifolium* were not commercially successful and overall resistance could not be transferred to new varieties due to its complexity.

Genetic analysis of resistance

The results of genetic studies with different resistance sources are summarized in Table 1. These studies showed that *Cmm* resistance is mostly polygenic [69, 54]. F_2 and backcross populations between resistant and susceptible *S. arcanum* accessions

have revealed the presence of two or three recessive genes involved in resistance [70]. An analysis of three resistant *S. lycopersicum* breeding lines (after crosses with resistant *S. chilense* and *S. peruvianum* accessions) identified a single dominant gene on Chromosome 4 with a few modifier genes [71]. Progeny plants obtained after crossing two moderately resistant *S. lycopersicum* parents showed higher levels of resistance than in either parent [6]. Transgressive segregation was also observed after *S. lycopersicum* and *S. peruvianum* resistance sources were crossed [67]. These studies were done without molecular marker technology. The resistance from *S. arcanum* LA2157 was studied in an intraspecific cross and five QTL regions on chromosome 1,6,7,8 and 10 were identified [72]. This same resistance source was used to make an interspecific cross and an F_2 population with *S. lycopersicum* and three QTLs located on chromosomes 5, 7 and 9 were identified. These resistance loci were additive and co-dominant and combining the main QTL on Chromosome 7 with one of the others gave a similar resistance level as the resistant parent *S. arcanum* LA2157 [73]. Another quite well characterized source was *S. habrochaites* LA407. Genetic analysis in an inbred backcross population (IBC) resulted in the identification of two QTLs (on chromosomes 2 and 5) [74]. Fine mapping of these QTLs resulted in a 4.4 cM interval on Chromosome 2 (Rcm 2.0) and a 2.2 cM interval on Chromosome 5 (Rcm 5.1). The interaction of the QTLs was additive [75]. Consequently different *Solanum* species contain different numbers of genes and generally it seems that *Cmm* resistance is polygenic. Resistance genes showing different types of interaction such as additive, incomplete dominance and modifying effect occur. Resistance type (dominance or recessiveness) even can change depending on the genetic background as for instance was evident from the resistance obtained from the *S. arcanum* LA2157 source [72, 73]. The interaction of resistance genes with different environmental conditions is a further complicating factor.

Morphological resistance

Stem morphology and the vascular system vary in tomato and its related wild species. A study [76] that was conducted on the vascular structure of wild tomatoes, domesticated tomatoes and populations derived from these species has revealed several characteristics that may play a role in the resistance mechanism. The vascular structure of the hypocotyl region can vary from square to circular. The time in early development required for the root to stem vascular transition is different in *S. habrochaites* LA407 compared to *S. lycopersicum*. In *S. habrochaites* and in cherry type tomatoes the time period is shorter. In addition, the primary vascular bundles and secondary vascular tissues are thicker in *S. habrochaites.* In an F_2 population of an inbred backcross line a significant association between markers and size of primary vascular bundle, shape of the vascular system, and thickness of secondary vascular tissue were found on Chromosome 2. Plants with this region homozygous *S. habrochaites* had longer primary vascular bundles, thicker secondary vascular tissue,

and triangular stem shape. Interestingly, this same region on Chromosome 2 has been found to be associated with *Cmm* resistance and with other morphological characters. Based on this study, we may assume that morphological differences between resistant wild species and processing tomato play a role in *Cmm* resistance. The shape and thickness of vascular bundles and a faster vascular growth results in stronger vascular tissue and this might play a role in resisting cell wall degrading enzymes produced by *Cmm*. In our studies we observed wilting symptoms in young *S. pimpinellifolium* plants that disappear in adult plants, while this was not observed in *S. lycopersicum* where during plant development more and more wilting occurred [15]. Temperature, plant age, resistance and inoculum concentration play a role in the incubation period and disease development. Generally, as plant age increases, the incubation period of *Cmm* also increases. With cooler temperatures, the incubation period increases and disease development is slower. The inoculum concentration (until a certain level) is negatively correlated with incubation period and positively with disease severity. Obviously a high resistance level of the host plant results in an increased incubation period and a decreased disease severity [77]. Infected plants with no symptoms and susceptible cultivars which are infected late in the season can be a source of *Cmm* infection for the next growing season. Farmers need to take serious precautions against any kinds of potential *Cmm* sources before starting a new growing season.

Table 1: Gene interaction types of different *Cmm* resistance sources

Resistance Source	**Population type**	**Gene(s) interactions**	**Reference**
S. lycopersicum *S. pimpinellifolium* *S. habrochaites*	*S. lycopersicum* lines Bulgaria 12, Homestead, Heinz 1350, Highlander and Campbell *S. pimpinellifolium* Utah 20 *S. habrochaites* PI251305	Polygenic and horizontal type resistance (regardless of strains)	[54, 69]
S. lycopersicum line Bulgaria 12	F_2 and backcross	Incomplete dominant genes with one to four major genes	[54]

S. lycopersicum lines Hawaii 7998 and Irat-L3	RIL population	Complementary genes with transgressive segregation	[6]
S. pimpinellifolium Utah 737 and Utah 20	F_2 and backcross of interspecific cross	The estimated gene number ranges from 4 to 11 with presence of modifying genes	[78]
S. peruvianum var. *humifusum*	F_2 and backcross population of resistant three-genome hybrid *S. lycopersicum* Line Cm 180 [*S. peruvianum* var. *humifusum* x (*S. lycopersicum* x *S. chilense* LA 460)]	A single dominant gene on Chr 4.	[71]
S. arcanum LA2157	F_2 and backcross of intraspecific cross	Two to three genes with recessive inheritance	[70]
S. arcanum LA2157	Backcross of intraspecific cross	5 regions on chromosomes 1,6,7,8 and 10	[72]
S. arcanum LA2157	F_2 population of interspecific cross	3 QTLs on chromosomes 5, 7 and 9. Additive interactions of QTLs	[73]
S. habrochaites LA407	Inbred backcross lines of interspecific cross	2 QTLs on chromosome 2 and 5. Additive interactions of QTLs	[74]

Bacteria movement and spread in the plant

Understanding the colonization of the tomato plant stem by *Cmm* is important to study resistance mechanisms. *Cmm* infection of plants can be caused by infected seeds (primary infection) or through agronomic practices (secondary infection) such as insufficient cleaning of greenhouses, clipping pruning and contact infections, human activity, rain and splashing etc. After infection, *Cmm* moves into the xylem vascular tissue where it can easily spread and where it can find a suitable place to

colonize. At the start of infection, *Cmm* moves to some areas of the tomato plant and the presence in infected plants is unequally distributed. Absence of *Cmm* in some parts of plants does not mean absence of *Cmm* in other parts of the plant [18]. Microscopic observations of *Cmm,* in the petioles of susceptible plants, five weeks after inoculation showed that the amount of bacteria increases in some parts of the xylem tissue [79]. Initially they multiply between spiral thickenings and later bacteria fill the xylem lumina. When bacteria end up in xylem vessels there is a rapid upward spread. This spread can also go to adjacent xylem vessels. The spread of bacteria into adjacent xylem bundles explains the one-sided wilting, a characteristic bacterial canker symptom. Subsequently, the bacteria start to attack primary cell walls of phloem tissue and a lateral spread occurs. After the lateral spread, *Cmm* destroys the xylem and subsequently phloem tissues, and multiplies. In contrast to the rapid movement of bacteria in the xylem, bacteria do not move freely in the phloem tissue because of the sieve tube structures there. When a susceptible tomato plant was inoculated in the roots, the bacteria spread in the plant in a similar fashion as they do after petiole inoculation: first to the xylem and later into the whole plant. A similar observation was made in another study [80]. Extracellular enzymes are thought to be responsible for the degradation of the primary wall and later middle lamella of xylem and phloem tissues. No vessel plugging material such as large amounts of plant degradation products, bacterial extracellular material or dense masses of bacterial cells were seen in infected xylem tissue. In the case of infected seeds the bacteria move, after germination, from the seed coat to the cotyledon and then further disease development takes place [81]. Our research [15] showed that although bacteria movement in the plant is unequal and unpredictable in the beginning of infection, at later stages bacteria are able to spread through the whole plant. Variation in the structure of the primary cell wall and the parenchyma cells of vascular elements may play a role in resistance to *Cmm*.

Pathogen side

On the bacterial/pathogen side a number of aspects are important in determining the disease occurrence and level. *Cmm* harbors two plasmids, pCM1 (27 kb) and pCM2 (70 kb), and a genome of 3,2 Mb [82]. Using deletion mutation and complementation, two genes involved in pathogenicity have been identified on the two plasmids, CelA on pCM1 [83] and Pat1 on pCM2 [84]. The CelA gene has a coding region of 2.4 kb encoding an Endo-β-1,4-glucanase, a protein of 78 kDa which consists of 3 domains; a catalytic domain, a type II like domain and C-terminal domain. A homolog of CelA lacking the third domain is present on the chromosome (CelB gene). The Pat1 gene putatively encodes a serine protease, a protein of 280 amino acids (29.7 kDa). It has two homologs on pCM2 (phpA and phpB) [85]. Protein of both genes have a signal peptide at the N terminus and are secreted [86].

A *Cmm* mutant lacking a chromosomal region of 129 kb resulted in impaired virulence and inability to effectively colonize a plant. Genome sequencing and annotation has revealed six homologous of the Pat1 gene. chpB-G (with two pseudo genes), chpB and chpD are putative genes coding for serine proteases located on the chromosomal PAI island [11]. In the PAI island chpC was the first identified gene that plays a role in the interaction of *Cmm* with its host [87]. A *Cmm* tomatinase gene (also located in the PAI region) is responsible for the breakdown of the secondary plant metabolite, α-tomatine. α-Tomatine is known to be a basal defense component of tomato [88]. Therefore this PAI of 129 kb was named chp/tomA region. Only in the absence of this chp/tomA region, basal defense genes were induced at an early stage of infection suggesting an involvement in suppression of basal host defense. The tomA sub-region seems not to be involved in pathogenicity [88], but it was suggested that this sub region makes it possible to utilize plant derived nutrients [89].

Transcriptional analysis of wild type *Cmm* and *Cmm* lacking both plasmids showed that there is an interplay of chromosomal and plasmid genes. Expression of celA and Pat1 on the plasmids was reduced in the absence of the PAI region, whereas expression of chpC and ppaA, which represent two different serine protease families, was reduced in the absence of the plasmids [90]. Interplay mechanisms are thought to be necessary for successful colonization by *Cmm*. *Cmm* lacking the chp/tomA region and one of the plasmids, pCM1 or pCM2, were not able to colonize a host effectively and only bacteria were found close to the area of inoculation [91].

Cmm is a xylem invading organism and in the xylem there is a low level of nutrients. The infection of *Cmm* starts biotrophic and *Cmm* is able to extract nutrients from poor environments. Fifty seven ABC transporter proteins have been found in infected plants [92]. When the *Cmm* population has reached a certain level *Cmm* changes its behavior and becomes necrotrophic and is secreting several enzymes. Proteins, belonging to the Ppa family (serine proteases) and the subtilase family play a role in plant colonization and disease development and were found in infected plants together with plant cell wall degrading enzymes such as pectate lyases and several glycosyl hydrolases including CelA proteins [92]. Genes encoding for extracellular enzymes which are necessary for successful invasion of plant tissue by degradation of xylem walls are up-regulated at early stages of infection [91] and later down-regulated [89]. The function of those genes, therefore, is thought to be the triggering of early signal cascades. Another putative virulence gene encoding a perforin protein might enable bacteria to manipulate host genes by delivering bacterium effectors into host cells. This mechanism is similar to the up regulation of type-III secretion systems after infection of gram-negative bacteria [89].

The transition of *Cmm* from biotroph to necrotroph is determined by the population size, the threshold is 10^8 cfu/g plant material [93]. Wilting starts at this threshold and is thought to be caused by vessel plugging and/or toxin action. However, no vessel plugging material (large amounts of plant degradation products, bacterial extracellular material or dense masses of bacterial cells) were found in *Cmm* invaded plants [80]. Therefore, the presence of toxins is more likely to be the cause of wilting. Although bacteria are single cells, their gene expression is influenced by cell population density (quorum sensing). Quorum sensing enables bacteria to become more effective [94]. The transition from biotroph into necrotroph might be the result of bacterial concentration in the host which might be a quorum sensing dependent phenomenon. Microorganisms produce extracellular polymeric substances (EPS), a complex mixture of biopolymers consisting of polysaccharides, proteins, nucleic acids, lipids and humic substances. EPS enable bacteria to attach to certain substrates and are a protection against environmental stress and dehydration [95]. EPS of *Cmm* consist of L-fructose, D-galactose, D-glucose, pyruvate, succinate and acetate in a ratio of 2:1:1:1:0.5:1.5 [96]. A non-virulent *Cmm* produces lower concentrations EPS with different compositions, and no hypersensitive response (HR) on *M. jalapa* is induced. Purified EPS from *Cmm* were able to induce wilting of tomato cuttings of both resistant and susceptible accessions [96] and strains producing only 10% of the level of EPS of a normal strain were as virulent as non-mutant strains, furthermore plasmid free *Cmm* didn't cause wilting even though they produced identical composition and quantities of EPS [97]. EPS is not thought to be involved in pathogenicity but is assumed to play a role in colonization [89]. The role of EPS in *Cmm*-host interaction has not been elucidated yet, EPS might be involved in colonization, through protection of bacterial cells by making a protective biofilm [91]. Candidate genes encoding surface proteins might be responsible for the production of such a biofilm [89].

The secondary plant metabolite α-tomatine, is known to be a basal defense component in tomato; it reduces pathogen population growth of fungi [98] and bacteria [99]. The level of α-tomatine rises after pathogen attack [99]. *Cmm* has a tomatinase gene (tomA) resulting in the breakdown of α-tomatine, however a *Cmm* tomA mutant strain was as virulent as the non-mutant strain. Other experiments showed that α-tomatine inhibited the growth of the mutant *Cmm* more than it did with the wild *Cmm* [88]. Although the role of α-tomatine is not clear in host defense, the quantity of α-tomatine might play a role in the basal or activated defense system.

Plant side

After infection, plants can recognize pathogens through a pathogen-associated molecular pattern (PAMP) mechanism. In the tomato-*Cmm* interaction, putative PAMP proteins were identified based on their up-regulation after infection. Several protein phosphatases which play a role in activating signal transduction cascades and several kinases which are known to be involved in defense mechanisms against bacteria were detected in the plant after *Cmm* infection [92]. Also the basal defense of the host plant was activated after infection. Basal defense includes defense related genes, production and scavenging of free oxygen radicals, enhanced protein turnover and hormone synthesis [92, 100]. Down regulation of some metabolic pathways such as photosynthesis and up-regulation of senescence-associated proteins [101] happen after infection. Gene expression studies [100] on different time points (4 days after inoculation but before symptoms were visible and 8 days after inoculation with the first wilting symptoms) revealed that in total 122 genes (of a total of 9,254) were differentially expressed on at least one time point. The majority of genes influenced by *Cmm* at an early stage were also differentially expressed in later stages of disease development. A significant induction of expression of ethylene synthesis genes was found after infection however without ethylene emission in the early stage of pathogenesis but in later stages ethylene was induced in the infected tomato stem. Ethylene insensitive never ripening (Nr) plants and ethylene deficient mutants have a significant delay in wilting so it appears that ethylene does play a role in disease progress but it has not yet been shown how [100]. The most abundant proteins that were found after infection with *Cmm* were enzymes involved in methionine metabolism and ethylene biosynthesis [92]. Since ethylene was thought to be involved in susceptibility [102], it is speculated that *Cmm* induces ethylene synthesis in tomato in early stages of infection which results in softening the vascular tissue by senescence making it more easy for *Cmm* to enter. One hundred and sixty genes which were down or upregulated during the response of tomato to *Pseudomonas syringae pv. tomato* were tested in *Cmm* inoculated *Nicotina benthamiana*. These genes were individually silenced and silencing of six genes, including StSN2 and ELP, resulted in significantly higher bacterial titer and faster wilting [103]. Overexpression of these two genes in tomato enhanced the tolerance to *Cmm* with significant delay of symptoms, reduction of lesion size at inoculation point and lower bacterial population in comparison to non-transgenic tomato [104]. The StSN2 gene encoding the snaking-2 (SN2) cysteine rich peptide is considered as antimicrobial gene and extension-like protein gene (ELP) encoding cell-wall hydroxylproline-rich glycoprotein are two important plant defense genes that were also found differentially regulated during the infection of tomato plant with *Cmm* [100].

A proteomic analysis of lines containing *Cmm* resistant QTL Rcm 2.0 and 5.1 and a susceptible line in response to *Cmm* infection at 72 and 144 h post-inoculation identified in total 42 differentially expressed proteins and 5 constitutively expressed proteins that could be further analyzed. Twenty-six of these proteins could be annotated. The accumulation of specific proteins was dependent on the genotype and on the post-inoculation time. The annotated proteins were involved in defense and stress response, protein regulation, protein synthesis and processing, energy production and metabolism. Lines with QTL Rcm 2.0 and 5.1 produce specific proteins and also reduce the *Cmm* population size somewhat which does not occur in the susceptible tomato line. It is thought that those QTLs respond to *Cmm* with different mechanisms [105].

After infection of tomato with *Cmm*, the pathogen moves to the xylem where it can spread and invade the whole plant. Most probably activation of basal defense system starts after *Cmm* changes its behavior from biotroph to necrotroph via quorum sensing and attacks xylem vascular tissue for lateral spreading and retrieving nutrients from phloem tissue. During this process, *Cmm* secretes proteins which changes the gene regulatory system of the host such as ethylene production. Possibly *Cmm* influences other plant metabolism systems so that it can easily break cell walls and disease progress can take place. By doing that the *Cmm* population size can increase.

Conclusion:

In this paper, we have discussed the biology, detection methods and host-pathogen interaction of *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) and tomato. The resistant plant sources which were identified were discussed. Understanding the *Cmm* interaction with tomato will provide us new perspectives for a better management of and maybe even combating this disease. Since *Cmm* strains are diverse, it is important to know by which mechanisms they attack and whether it differs from strain to strain. Understanding host responses to *Cmm* which possible varies in different tomato species, will give us insights why plants are resistant. Wild tomato species may be able to interfere with *Cmm* attack strategies. Speeding up growth rates after pathogen infection might be other mechanisms in wild species. Both general concepts of host-pathogen interactions as specific studies into the interaction of *Cmm* and tomato will be essential to find gene(s) involved in resistance. Hopefully this will lead to tomato varieties resistant to *Cmm*.

Chapter 3

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

Accepted for publication

Euphytica 2012 : 1-9

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

Yusuf Sen[1,3], Zhu Feng[1], Henri Vandenbroucke[1], Jan van der Wolf[2], Richard G.F. Visser[1] and A.W.(Sjaak) van Heusden[1,4]

(1) Department of Plant Breeding, Wageningen University and Research Centre, PO Box 386, 6700 AJ Wageningen, The Netherlands.(2) Plant Research International Biointeractions and Plant Health, Droevendaalsesteeg 1, 6708 PB, Wageningen, The Netherlands (3) Graduate School Experimental Plant Sciences (4) Corresponding author, Email: sjaak.vanheusden@wur.nl.

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. Worldwide, *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: *S. pimpinellifolium* GI.1554, *S. parviflorum* LA735 *and S. parviflorum* LA2072. We also confirmed the tolerance which was reported previously in *S. arcanum* LA2157, *S. arcanum* PI127829, *S. arcanum* 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*.

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% [6]. 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 [4]. 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 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 [106]. 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 [refs in 54]. Later, other tolerant accessions were reported such as *Solanum habrochaites* [55-58], *Solanum lycopersicum* [60, 62, 64, 65] and *Solanum S. arcanum* [57, 107]. Some of these tolerant sources were used for introgression breeding such as Bulgaria 12 [60], Heinz 2990 [62], Okitsusozai 1-20 [64]. These varieties were tolerant and showed partial wilting but still had a high bacterial titer. Tolerant sources [73, 58] were also containing high bacterial levels. Breeding for *Cmm* tolerant cultivars is difficult due to the complex inheritance, that is both polygenic and additive.

Cmm is internationally a quarantine organism [108] 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 on 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 reactions 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 to 7 days to get results. DNA based methods, like TaqMan real time PCR assays, are fast, sensitive and highly specific and it allows quantification. TaqMan assays have been used to identify Clavibacter subspecies [46] and to identify *Cmm* in seeds [109]. 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 [47]. 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 have formed to colonies will be detected. TaqMan assays have not been used for quantification of *Cmm* 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.

Material and Methods

Plant material

Twenty four wild species of tomato (Table 1) including reported tolerance sources *S. arcanum* LA2157, LA385, LA334, *S. habrochaites* LA407 and *S. lycopersicum* IRAT L3 were screened for tolerance to *Cmm*. *S. lycopersicum* cv Moneymaker was added to the screening as susceptible control. Each accession was represented with five 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 5µl of 10^8cfu/ml bacterial suspension in the wound. Approximately 10 cm above the first inoculation (between the fourth and fifth leaves), a second inoculation was done by injecting another 5µl bacterial suspension. After inoculation, plants were kept for one week under high relative humidity (100%) and subsequently at 60% humidity, 12 hours 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 less than 50% leaves are wilted, score = 2; between 50-75% 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) [110] and D_2ANX [111] were used to quantify bacteria accurately. Accessions representing three different resistance groups 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 (3 times the weight of the stem part). For the selective media, 100 µl from 10^4, 10^5, 10^6–fold dilutions were plated in three fold and remaining parts of original extraction from each plant belonging to each accession was mixed regardless of origin then this extraction was used for a 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. Five µl of this suspension was diluted with 45µl 5mM NaOH solution. This suspension was used as template in the PCR reaction. Primers were chosen based on pCM1, *CMM*3-4 [112] and pCM2, P5-6,[37] and the reaction was done in 50µl total reaction volume (5µl PCR buffer solution (10x), 0.2µl Taq polymerase (5U), 1µl 5mM Deoxynucleoside triphosphates (dNTPs), 2.5µl (25pmol) primer 1 and primer 2.5µl of DNA template and 33.8µl dH_2O PCR conditions were 35 cycles of 94 ºC - 30 sec; 60 ºC - 30 sec; 72 ºC 45 sec. 15µl 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 combination with a Kingfisher processor and followed by a purification step on a PVPP column. The 25 µl reaction includes 10 µl DNA template, 12.5 µl 10x Takara mix, 0.5 µl Rox and 2 µl mix of 4 µM Forward primer (GGG GCC GAA GGT GCT GGTG), 4 µM Reverse primer (CGT CGC CCG CCC GCTG) and 1 µM TaqMan probe with some modification (6-FAM/TGG TCG TCC /ZEN/TCG GCG CC/IABkFQ) [113]. TaqMan probe is based on a chromosomal region of the *Cmm* sequence. The real-time PCR temperature regime was as follows: 95 ºC for 30 s followed by 50 cycles of 95 ºC for 3 s and 60 ºC for 35 s using Bio-Rad CFX thermocycler. To obtain a standard curve, 3 independent replication of ten-fold 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. DNA extractions were done as described above. The standard curve is shown in Figure 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 [114] provided by Wageningen University, Plant Research International Biointeractions and Plant Health group. 25 µl (containing 5 µl sample DNA) or 30 µl

(containing 10 µl sample DNA) PCR solution consisted of 12.5 µl 10x Takara mix, 0.45 µl Rox and 2 µl of TaqMan primer's mix (4 µM forward, 4 µM reverse and 1 µM probe), 0.45 µl of 5 µM GFP forward, 0.45 µl of 5 µM GFP reverse and 0.3 µl of 5 µM GFP probe, 0.8 pg GFP DNA and 2 µl dH_2O.

Statistical Analysis

To determine the correlation of three different methods for quantification of *Cmm* populations, data of each accession were averaged for each method and transformed to a log10 base mode. After data transformation, data of each method were plotted against another method to see the distribution of data then correlation between methods was done (with SPSS). To check differences between accessions, an 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 the 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).

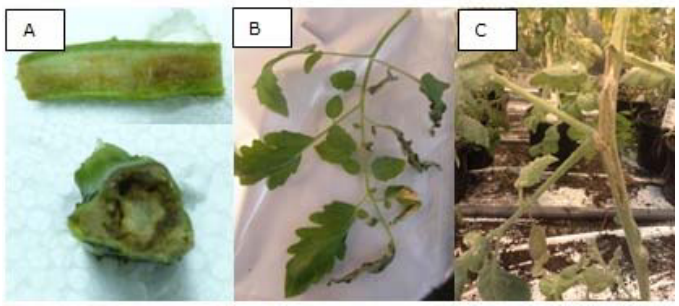


Figure 1: Typical disease symptoms of: stem discoloration (A), unilateral wilting (B) and stem canker (C)

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 (figure 2A).
Group 2: Accessions with a wilting score 2 with severe stem canker symptoms (figure 2B).
Group 3: A high level of wilting; score 4 and 5 (figure 2C).

Figure 2: The effect of bacterial canker on tomato: tolerant-no damage (A), moderately tolerant - middle damage (B) and susceptible -dead plant (C).

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

Accession name	**Disease Score**	**Tolerance level**
S. arcanum LA 2157	1	Group 1 (tolerant)
S. pimpinellifolium GI 1554	2	
S. arcanum LA 385	2	
*S. lycopersicum cv.*IRAT L3	2	
S. arcanum PI 127829	2	
S. parviflorum LA 735	2	
S. parviflorum LA 2072	2	
S. glandulosum IVT 63102	2	Group2 (moderate)
S. minutum CGN 15816	2	
S. glandulosum EC 495	2	
S. peruvianum LA 2334	3	
S. habrochaites LA 407	3	
S. habrochaites glabratum GI 1561	3	
S. chilense IVT 56140	3	
S. glandulosum IVT 48090	3	
S. minutum LA 1045	3	

S. parviflorum LA 2133	3	
S. chilense IVT 56139	4	Group 3 (susceptible)
S. lycopersicum cv Moneymaker	5	
S. pennellii LA 716	5	
S. habrochaites LYC4	5	
S. cheesmanii LA 1401	5	
S. cheesmanii LA 0166	5	
S. cheesmanii LA 1448	5	
S. cheesmanii LA 1409	5	

Detection and quantification of *Cmm* on selective media

On the semi-selective media D_2ANX 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_2ANX 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 10^2 to 10^8 cfu ml^{-1} and Ct values (threshold cycle value) is shown in figure 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 10^3cfu/ml, below this threshold bacteria are still detectable but it is 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 µl DNA sample DNA. In case of standard dilutions and samples, the 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.

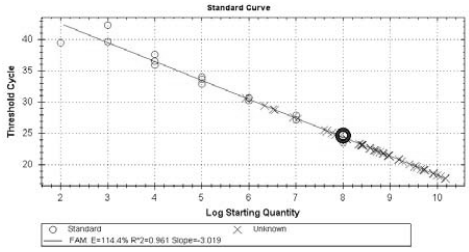


Figure 3: Detection and quantification of *Cmm* by TaqMan-PCR. A ten-fold serial dilution was tested in three replicates and the Ct values are plotted against the log of the bacterial concentration. A semi-log regression line plot of the Ct value is shown versus the log of the bacterial densities.

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_2ANX 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* LA735 and *S. parviflorum* LA2072 accessions has not been reported before. We have confirmed a high tolerance in *S. arcanum* LA 2157, *S. peruvianum* PI 127829 and *S. arcanum* LA385 [68], and a moderate tolerance in *S. habrochaites* LA 407 [58] and *S. lycopersicum* cv. IRAT L3 [65]. The accession *S. arcanum* LA2157 [73] 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* [76]. *S. 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 [8]. Because of that, at least two different mediums are advised to quantify *Cmm* in plant material [44, 115]. In our study, the densities of cfu of *Cmm* on the two semi-selective media, D_2ANX and SCM-fast, were measured and the numbers were generally higher on D_2ANX. We observed some colonies of saprophytes on media D_2ANX 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 in *Cmm* is not stable [106]. Since *Cmm* with no plasmids were a small proportion of the population, we ignored their effect on disease score. Also repeated experiments on the most resistant accessions, *S. pimpinellifolium* GI.1554 and *S. arcanum* LA2157, 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* [48]. Although this is a reliable method it is very laborious and it takes 5 to 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 10^3cfu/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 to 1000 fold more bacteria than the tolerant sources, but the bacterial concentration among tolerant accessions varies. This might be due to different resistance mechanisms and the fact that a lack of symptom expression is not only based on a reduction of bacteria. [105]. 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 [116]. 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 order 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. arcanum* 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 quantification of *Cmm* in stem-inoculated accessions of tomato with a different level of susceptibility.

Acknowledgement:

We would like to thank Patricia van der Zouwen and Marjon Krijger from Wageningen University Plant Research International Biointeractions and Plant Health group for help, and the Scientific and Technological Research Council of Turkey (TÜBİTAK) for financial support.

Table 2: Comparison of bacterial concentration with three different methods: dilution plating on the selective media SCM-fast and D_2ANXand TaqMan-PCR. Upper is 30 cm above and lower is 10 cm below the inoculation point.

		Bacterial titer (cfu/g)		
Accessions	**Plant part**	**SCM-fast**	**D_2ANX**	**TaqMan (Average)**
S. lycopersicum cv.MM	Upper Inoculation Lower	$4.00x10^{10}$ $4.22x10^{11} \pm 4.19E+00^{a}$ $5.34x10^{11}$	$4.60x10^{10}$ $5.57x10^{11} \pm 4.83E+00^{a}$ $8.47x10^{11}$	$7.2\ x10^{10} \pm 2.64E+00^{a}$
S. arcanum LA 2157	Upper Inoculation Lower	$1.83\ x10^{7}$ $3.08\ x10^{8} \pm 6.10E+00^{bc}$ $5.33\ x10^{8}$	$3.30\ x10^{7}$ $3.87\ x10^{8} \pm 4.75E+00^{c}$ $5.90\ x10^{8}$	$5.21\ x\ 10^{8} \pm 7.24E+00^{bc}$
S. habrochaites LA 407	Upper Inoculation Lower	$1.32x10^{9}$ $2.81x10^{9} \pm 1.82E+00^{b}$ $8.63x10^{8}$	$3.10x10^{9}$ $3.39x10^{9} \pm 1.57E+00^{b}$ $1.48x10^{9}$	$1.41\ x\ 10^{9} \pm 4.11E+00^{bc}$
S. lycopersicum cv. IRAT L3	Upper Inoculation Lower	$9.33x10^{8}$ $5.53x10^{8} \pm 1.43E+00^{bc}$ $1.10x10^{9}$	$1.32x10^{9}$ $1.26x10^{9} \pm 1.20E+00^{b}c$ $1.77x10^{9}$	$1.51\ x\ 10^{9} \pm 1.75E+00^{abc}$
S. pimpinellifolium GI1554	Upper Inoculation Lower	$4.20x10^{8}$ $5.74x10^{8} \pm 1.34E+00^{bc}$ $3.21x10^{8}$	$1.34x10^{9}$ $1.59x10^{9} \pm 1.10E+00^{bc}$ $1.37x10^{9}$	$1.79\ x\ 10^{9} \pm 2.45E+00a^{bc}$
S. arcanum PI 127829	Upper Inoculation Lower	$1.20x10^{8}$ $1.05x10^{8} \pm 1.07E+00^{bc}$ $1.12x10^{8}$	$1.39x10^{8}$ $1.39x10^{8} \pm 1.05E+00^{c}$ $1.28x10^{8}$	$1.50\ x\ 10^{8} \pm 6.92E+00^{c}$
S. parviflorum LA 735	Upper Inoculation Lower	$4.99x10^{8}$ $7.70x10^{8} \pm 1.38E+00^{bc}$ $9.41x10^{8}$	$7.80x10^{8}$ $1.08x10^{9} \pm 1.29E+00^{bc}$ $1.28x10^{9}$	$3.19\ x\ 10^{9} \pm 3.59E+00^{abc}$
S. parviflorum LA 2072	Upper Inoculation Lower	$9.13x10^{8}$ $6.95x10^{8} \pm 2.23E+00^{bc}$ $2.03x10^{8}$	$1.01x10^{9}$ $7.75x10^{8} \pm 2.21E+00^{bc}$ $2.27x10^{8}$	$3.79\ x\ 10^{9} \pm 3.20E+00^{ab}$
S. arcanum LA 385	Upper Inoculation Lower	$2.07x10^{8}$ $7.47x10^{7} \pm 2.07E+00^{c}$ $5.03x10^{7}$	$2.70x10^{8}$ $1.44x10^{8} \pm 1.50E+00^{c}$ $1.27x10^{8}$	$2.46\ x\ 10^{8} \pm 6.33E+00^{bc}$

*Different letter indicates significant differences between accessions

Chapter 4

QTL mapping of *Clavibacter michiganensis subsp. michiganensis* (*Cmm*) resistance originating from *Solanum pimpinellifolium* G1.1554

Submitted for publication

QTL mapping of *Clavibacter michiganensis subsp. michiganensis* (*Cmm*) resistance originating from *Solanum pimpinellifolium* G1.1554

Yusuf Sen[1,4], Mary Jane Manrique[1], Aylin Kabaş[2], Marcos Malosetti[3], Richard G.F. Visser[1], A.W.(Sjaak) van Heusden[1,5]

(1) Wageningen UR Plant Breeding, Wageningen University and Research Centre, PO Box 386, 6700 AJ Wageningen, The Netherlands (2) West Mediterranean Agricultural Research Institute, Demircikara Mh. PaşaKavakları Cad. N: 11, Muratpaşa Antalya, Turkey (3) Biometris, Wageningen University, PO Box 100, Wageningen, 6700 AC, The Netherlands (4) Graduate School Experimental Plant Sciences, Wageningen Campus, 6708 PB Wageningen, The Netherlands (5) Corresponding author, Email: sjaak.vanheusden@wur.nl

Abstract

Bacterial canker of tomato, caused by *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), is considered the most serious bacterial threat in tomato and causes large damages in production areas. Worldwide, *Cmm* is subjected to quarantine regulations. There is no cultivar on the market containing *Cmm* resistance. A mapping study was done in order to identify Quantitative Trait Loci (QTL) for resistance in a cross between *S. lycopersicum* and *S. pimpinellifolium* GI.1554, a wild relative of tomato. Besides wilting, symptoms like stem discoloration and bacterial titer were considered. Using single trait and multi-trait approaches, we have identified five QTL regions that are associated with wilting, stem discoloration and bacterial titer in three different environments. These QTLs can be used in breeding programs to develop cultivars with higher levels of resistance.

Keywords: Clavibacter, *S. pimpinellifolium*, Infinium array, Multi-trait analysis, QTL to candidate genes

Introduction

Clavibacter michiganensis subsp. michiganensis (*Cmm*) is a gram positive plant bacteria causing bacterial canker in tomato and is considered to be the most harmful bacteria in tomato [3]. Symptoms appear as unilateral wilting and at a later stage the whole plant wilts and dies. Besides wilting, stem canker and stem discoloration are symptoms of this disease. *Cmm* is a seed transmitted disease and even a few infected seeds (one to five seeds per 10,000) can result in a serious epidemic in the field [17]. *Cmm* is considered as a quarantine organism and seed companies have to take special precautions to prevent the presence of

Cmm to be able to sell seeds. A good level of *Cmm* resistance might reduce some of the problems but there are two kinds of demands to be met: those of the breeders and those of the growers. Breeders would like to have a resistance source that does not allow the growth of a single bacterium in order to be able to sell their seeds. However, for growers, this is not an absolute requirement and a cultivar with a good resistance level, but still some bacterial growth, might already be sufficient and will be a kind of insurance that losses due to a *Cmm* outbreak will not be significant. *Cmm* is not considered a serious disease by some breeders because they think that good clean management practices will prevent outbreaks, but still in practice outbreaks occur continuously (personal communication with growers) and new varieties containing a good level of *Cmm* resistance certainly have an added value in conventional and organic farming. Breeding for bacterial canker resistance in tomato is already going on for almost 50 years [60]. A partial resistant variety has been described [62] but this resistance has never been used in the development of new partial resistant varieties. Without sufficient resistance in tomato varieties it was necessary to screen crossable wild species of tomato for resistance [59, 117]. After identifying resistance in *Solanum arcanum* LA2157 a genetic analysis has been elaborated using intra- and interspecific crosses. In the intraspecific cross five QTL regions on chromosomes 1,6,7,8 and 10 were identified [72] and the interspecific crosses revealed 3 QTLs on chromosomes 5,7 and 9 [73]. In other interspecific crosses between *Solanum lycopersicum* and *Solanum habrochaites* LA407 at least 2 QTLs, on chromosomes 5 and 7, were found [74].

Wild species of tomato are a good source to enlarge the genetic diversity in the gene pool of commercial tomato. In a screening for resistance [15] a *Solanum pimpinellifolium* (G1.1554) accession was found with a good resistance level. *Solanum pimpinellifolium* is a closely related wild species of tomato and is easily crossable with cultivated tomato [114]. Finding resistance genes/QTLs in different sources will make it possible to combine genes from different sources with possible different mechanisms which might give a higher level of resistance. A good phenotyping method, a sufficiently large population with ample opportunities for reproducing screenings and a high density linkage map are the necessary tools to do good mapping studies. To know the QTL x environment effect, it is necessary to do screenings in different environments.

We have used a very well genotyped recombinant inbred line (RIL) population derived from *Solanum lycopersicum* cv Moneymaker and *Solanum pimpinellifolium* G1.1554 [118].For bacterial quantification, we have used a TaqMan PCR assay [15]. The RIL population was phenotyped in three different environments; namely Dutch greenhouse in winter, Dutch greenhouse in summer and in a greenhouse in spring in Antalya, Turkey. QTL hot spots and QTLxE interactions for several traits were found.

Materials and Methods

Plant material and environments

One hundred recombinant inbred lines derived from a cross between *S. lycopersicum* cv. Moneymaker and *S. pimpinellifolium* GI.1554 were used for resistance screenings. We screened in three different environments (Table 1). The first environment was a greenhouse in winter in the Netherlands where outside conditions are cold (-10 to 10 °C), cloudy and short days. The second environment was a greenhouse in Dutch summer when it is relatively warmer (10 to 25 °C) and long days that might even be sunny. A third screening was done in a greenhouse in Antalya, in the south of Turkey where the growing season is from January to May with temperatures varying from 5 to 30°C and at least half of the season is sunny. In the first and second screening, conditions in the greenhouse were controlled (18°C at night and 24°C during the day with 60% humidity) whereas in the third environment this was not the case. In the first and second environment, each line and parents were represented by 4 plants, in the third environment each line and parent was represented by 8 plants and 2 control plants.

Disease test

In the Netherlands we have used the aggressive bacterial strain *Cmm*542, in Turkey we have used a mix of 14 local aggressive strains of which specificity is not known. Inoculation was done at the sixth leaf stage by removing the second leaf with scissors and injecting 5µl of 10^6 cfu/ml bacterial suspension in the wound. In the first and second environment, after inoculation, plants were kept for one week under high relative humidity (100%), then conditions were changed to 60%, 12 hours daylight, 24°C day and 18°C night temperature. In Turkey (3rd environment) plants were kept under greenhouse conditions after inoculation. Wilting symptoms were recorded using the following scale: 0.5 stands for 12.5% wilting, 1 for 25% and scale continues until 100% wilting. No symptoms, score = 0 and when the whole plant is wilted and dead, score = 4.The final wilting symptoms were recorded at 65 days after inoculation. Stem discoloration was measured on the inoculation area of the stem after the experiment finished based on following scale: 0.5 scale was used for each 12.5% discoloration stem clean, score = 0; and stem is totally rotten, score = 4.

Quantification of bacteria by TaqMan PCR

To quantify bacteria, three plants from each line and 3 parts of each plant, hereafter referred to as lower, middle part and upper part, were used. The extraction of bacteria from this material was done using PBS buffer (3 times the weight of the stem part). DNA extraction was done using the Quick Pick SML Plant DNA purification kit provided by Bio-Nobile in combination with a Kingfisher processor and followed by a purification step on a PVPP column. RT-PCR amplification was done as follows; The 25 µl reaction includes 10 µl DNA template, 12,5 µl 10x Takara mix, 0,5 µl Rox and 2 µl mix of 4 µM Forward primer

(GGG GCC GAA GGT GCT GGTG), 4 µM Reverse primer (CGT CGC CCG CCC GCTG) and 1 µM TaqMan probe (modified) (6-FAM/TGG TCG TCC /ZEN/TCG GCG CC/IABkFQ) [113]. The real-time PCR temperature regime was as follows: 95 ºC for 30 seconds followed by 50 cycles of 95 ºC for 3 seconds and 60 ºC for 35 seconds using a Bio-Rad CFX thermocycler. To obtain a standard curve, 3 independent replications of ten-fold serial dilutions of bacteria were used as a template and a water control was included as negative control.

Genetic map

Custom-made Infinium Bead arrays containing 5528 SNPs were used for genotyping the population [119]. A genetic map was constructed using Joinmap4.1 software [120] using a regression algorithm with Kosambi mapping function.

QTL analysis

Data were analyzed by two approaches; single trait single environment and multi trait single environment. Single trait single environment analysis of data was done by MapQTL6.0 software [121] using interval mapping. In order to convert scale type data to continuous style data that allow interval mapping, data were transformed to log scale prior QTL analysis. The Q-Q plot test was used to inspect the distribution of residual data. For interval mapping, a permutation test (10,000 times) was done to determine the genome wide threshold for QTL detection. The logarithm-of-odds (LOD) profiles from interval mapping were inspected and the marker closest to each LOD peak was selected as cofactor and the backward elimination procedure was used to select the significant cofactors. This backward elimination procedure was performed until stable cofactor subsets had been obtained. Remaining cofactors were used for further rMQM mapping analysis. For multi-trait single environment analysis, data were standardized according to formula: $X_A = (x-\bar{x})/SD$. Here each value is subtracted from the mean and divided by the standard deviation. We have used a multi-trait single environment model per environment using GenStat version 14.0 [122]. A mixed model composite interval mapping algorithm was used to detect QTLs assuming QTLs as fixed effects in the model, and an unstructured variance covariance model for the residual multi-normal polygenic effect. Details about models and methods can be found in [122].

Heritability Estimates

Total genotypic variance were obtained from a one-way random effects analysis of variance using GenStat version 14.0 [122]. Total variance was partitioned in two components; variations between lines (Vg) and variation within lines, or error variance (Ve). Broad-sense heritability was (H^2) estimated using both variances according to the formula; $H^2 = Vg/(Vg + Ve/n)$ n= number of replicates.

Retrieving candidate genes

We have used the Marker2 sequence program [123] to retrieve candidate genes in the QTL hot spot region.

Results

Wilting symptoms of the overall population were different in each environment. Under Dutch controlled greenhouse conditions and the use of a single aggressive strain, there was a tendency towards higher susceptibility in the winter screenings compared to the summer screenings (Fig. 1, blue vs green). Under uncontrolled conditions in Antalya only 7 lines were partially resistant (Fig. 1, red). In the Dutch screenings, the susceptible parent was always the first genotype that totally wilted and the resistant parent showed the highest resistance level. No transgressive segregation was observed for wilting in Dutch winter and Dutch summer environments. In Antalya, where growing conditions were poor and a mix of strains was used, transgressive segregation was observed for wilting and the resistant parent showed only a moderate level of resistance. The bacterial concentration in Dutch winter and summer varied between 10^6 and 10^{10}. A large and significant contrast was found between the parents (10 fold).

The number of available, polymorphic SNP markers between *S. lycopersicum* and *S. pimpinellifolium* was 2497. After removing all but one of the identically segregating loci and markers with a poor goodness-of-fit in the map, we were able to create a genetic linkage map containing 870 SNP markers in 17 linkage groups corresponding to 12 tomato chromosomes. The total genetic size of our map was 1320 cM and large differences were found in recombination frequencies on chromosomes.

The heritability of the different traits were high, between 0.6 and 0.8, except for the bacterial titer in the upper part in Dutch Summer (h^2=0.43). The correlation between traits varied from 0 and 0.8. In general, a moderate correlation was found ranging from 0.3 to0.4 (Table 3).

A single trait QTL analysis yielded in total 7 QTL (Figure 2). No QTL was detected for stem discoloration in any environment and no QTL was detected for wilting in Dutch summer. The explained variance of these QTL varied from 12.6 to 34.9 (Table 4).

The multi-trait approach identified five regions on five different chromosomes (Chr1, Chr2, Chr7, Chr8 and Chr12) with potentially multiple QTL per region. At most 18 QTLs were identified by this approach. The multi-trait approach detected additional QTL regions on chromosomes 1, 2 and 8 and the explained variance for QTL that were detected varied from 4.5 to 32.5 (Table 4).

For the major QTL on Chromosome 7 we used the physical position of the SNP markers flanking the QTL (ch07:60289256...61494964) and searched in this 1.2 Mb area for genes. In

total 157 genes were found of which 5 belong to the NBS-LRR disease resistance class which might play role in resistance.

Discussion

Dissecting phenotypic responses into different components makes it possible to understand the mechanisms of resistance better [124]. We have dissected our disease response in three components; wilting, stem discoloration and bacterial titer. To prevent false positive and false negative results, the bacterial titer has been measured in three different parts of the plants. Environmental effects influence the severity of disease symptoms. Plants appear more resistant in conditions where they can grow well (Fig. 1). Based on the involvement of multiple loci and the large GxE effects, we assume that the resistance mechanisms are both morphological and physiological. Our hypothesis is supported by previously published research [76, 125].

The SNP Infinium array enabled us to construct a high quality genetic map. Genetic order and physical order of markers was very consistent. The total genetic map size was similar to the Kazusa Map [126]. Cold spots (low recombination rate) were detected in large regions around the centromere and hot spots (high recombination rate) were found outside the centromeric region where genes are more abundant and less repetitive DNA is present [127]. Due to a lack of markers in the recombination hot spot areas, some chromosomes were represented by more than one linkage group. Due to the known positions of the SNPs, it is possible to look for other SNPs if needed for fine mapping purposes. The genetic dissection of *Cmm* resistance in a recombinant inbred line population has revealed several QTL for *Cmm* related traits. Previously reported publications also show that several regions were involved in resistance [54, 72-74]. The severity of wilting is generally considered as the best indication of resistance. Multi-trait QTL mapping using the three typical *Cmm* symptoms (wilting, stem discoloration and bacterial titer) showed that QTL are co-localized in regions of chromosome 1,2,7,8 and 12. Multi-trait analysis improved the power of analysis and identified additional QTLs on chromosome 1,2 and 8 but didn't confirm the QTL on Chromosome 2 (Dutch winter wilting) which was detected by the single trait approach.

In general, no QTL with a consistent effect in all environments for all three traits was found. The multi-trait approach revealed that the QTL on Chromosome 7 is stable across the environments for three traits (Table 4). A combination of the QTLs on chromosome 2 (multi-trait analysis) and Chromosome 7 (single and multi-trait analyses) gave a similar wilting as in the resistant parent (data not shown). The QTL on Chromosome 2 was not in the same region as a previously published QTL on Chromosome 2 originating from *S. habrochaites* LA407 [75]. The genomic region on Chromosome 7 with several QTL is located quite far from the QTL region which was found on Chromosome 7 that was designated as the most important QTL originating from *S. arcanum* LA2157 [73]. The heritability was generally high

for all traits (0.43 to 0.87), and the sum of the explained variances of the detected QTL didn't explain the whole heritability. This missing heritability can be due to missing QTL and noise in phenotyping. The epistatic interactions of QTL might also be cause of missing heritability [128, 129]. In the Dutch winter and summer environments, one aggressive strain was used whereas in Antalya a mix of 14 different aggressive strains has been used. Therefore, an effect of strain differences in experiments must be considered if gene-to-gene interactions exist. However gene-to-gene interactions are not expected in the *Cmm*-tomato interaction [86] thus we ignored the strain effect in our experiment which might have been involved in the instability of QTL across environments.

Multi-trait model QTL analysis takes into account the correlation of traits, in doing so the power of detecting QTL and precision of QTL position can be increased [130, 131]. Multi-trait interval analysis can detect QTL for traits in situations when the heritability is relatively high and the effect of traits is too small to allow detection by single-trait interval mapping [132]. Traits that were scored in our study are phenotypic responses to bacterial attack, and most probably identical pathways are involved resulting in parameters that are related such as bacterial titer and wilting. Multi-trait analysis can then be better than a single trait analysis. An example are the QTL which were detected on chromosomes 2 and 11 (Fig. 2). Sometimes the use of multi-trait analysis results in that QTL that were found with the single trait analysis are not found back. An example of this is the QTL for wilting on Chromosome 2. Differences in findings of QTL with the same data can be the result of the different statistical models involved and/or the inference methods used (single trait QTL mapping is based on a mixture model approach whereas multi-trait analysis is based on a mixed model approach).

QTL for three components (wilting, stem discoloration and bacterial titer) were found in several common regions (Table 4) which is an indication for pleiotropy or strong linkage. Fine mapping or QTL cloning may separate these two phenomena. For bacterial titer and wilting, transgressive segregation was observed. Transgression for the level of *Cmm* resistance has been reported before [6, 67]. Transgressive segregation was only observed under Antalya conditions. It is generally accepted that transgression is complementary gene actions which can be visible in recombinant individuals [133] plus with recessive alleles which came to homozygous state in this population [134]. Although dominance or over dominance are also thought to be involved in transgression [133] this was not the case in the RIL population since it has almost complete homozygous state. It seems that this transgression has strong environmental effect. The mechanism(s) underlying *Cmm* resistance in tomato are still largely unknown. In QTL 7 region, we have identified 5 NBS-LRR genes which might contribute resistance in quantitative respect by residual effect of R gene concept [135]. In the near future we will analyze the resequenced *S. lycopersicum* cv Moneymaker, *S. pimpinellifolium* and 60 of the RILs. This might give hints about gene(s) that are involved in the resistance and may give possible mechanisms perspective based on their known or putative function.

In conclusion, *Solanum pimpinellifolium* GI.1554 can be a good source for *Cmm* resistance. Other traits than wilting can be considered for screening in order to understand resistance mechanisms better and to identify the most useful and stable QTL. In this study, we have used a well-studied population and QTL analysis was done by two different approaches. Our conclusion is that multi-trait analysis was more powerful than a single trait QTL analysis. Nearly isogenic lines can confirm the effect of those regions in a *Solanum lycopersicum* background and fine mapping in the QTL hot spots, especially Chromosome 7, might point to candidate genes which makes it possible to understand the resistance mechanisms better. The QTL from *S. pimpinellifolium* can be combined with QTL from *S. arcanum* LA2157 in order to obtain higher levels of resistance, which are especially important for growers to prevent devastating outbreaks of *Cmm*.

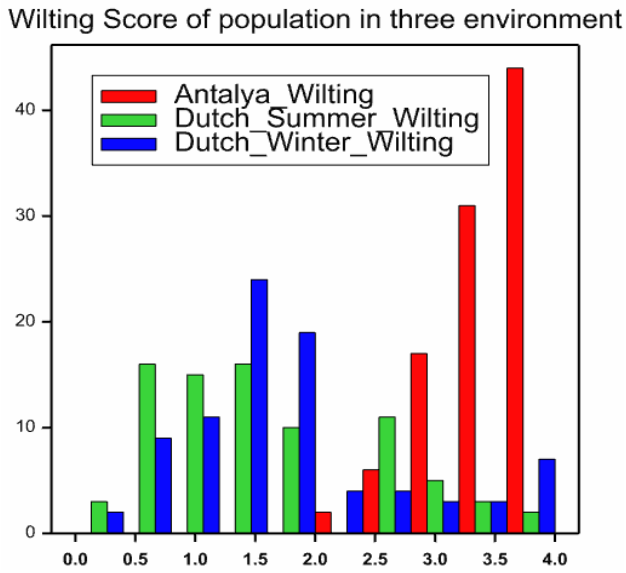


Figure 1. The distribution (before transformation) of wilting in three different environments. X-axis shows disease score and Y-axis the number of lines for each disease score.

Table 1. Screening conditions and scored phenotypic traits of the recombinant inbred population.

Screening conditions				**Measured traits**		
Environments	**Population size**	**Growing conditions**	**Bacterial strain**	**Wilting**	**Stem dis- coloration**	**Bacterial titer**
Dutch winter	100	Soilless	*Cmm* 542	done	done	done
Dutch summer	80	Soilless	*Cmm* 542	done	done	done
Antalya	100	Soil	Mix of strains	done	not done	not done

Table 2. Genetic map based on a RIL population derived from a cross between *Solanum lycopersicum* cv. Moneymaker and *Solanum pimpinellifolium* G1.1554

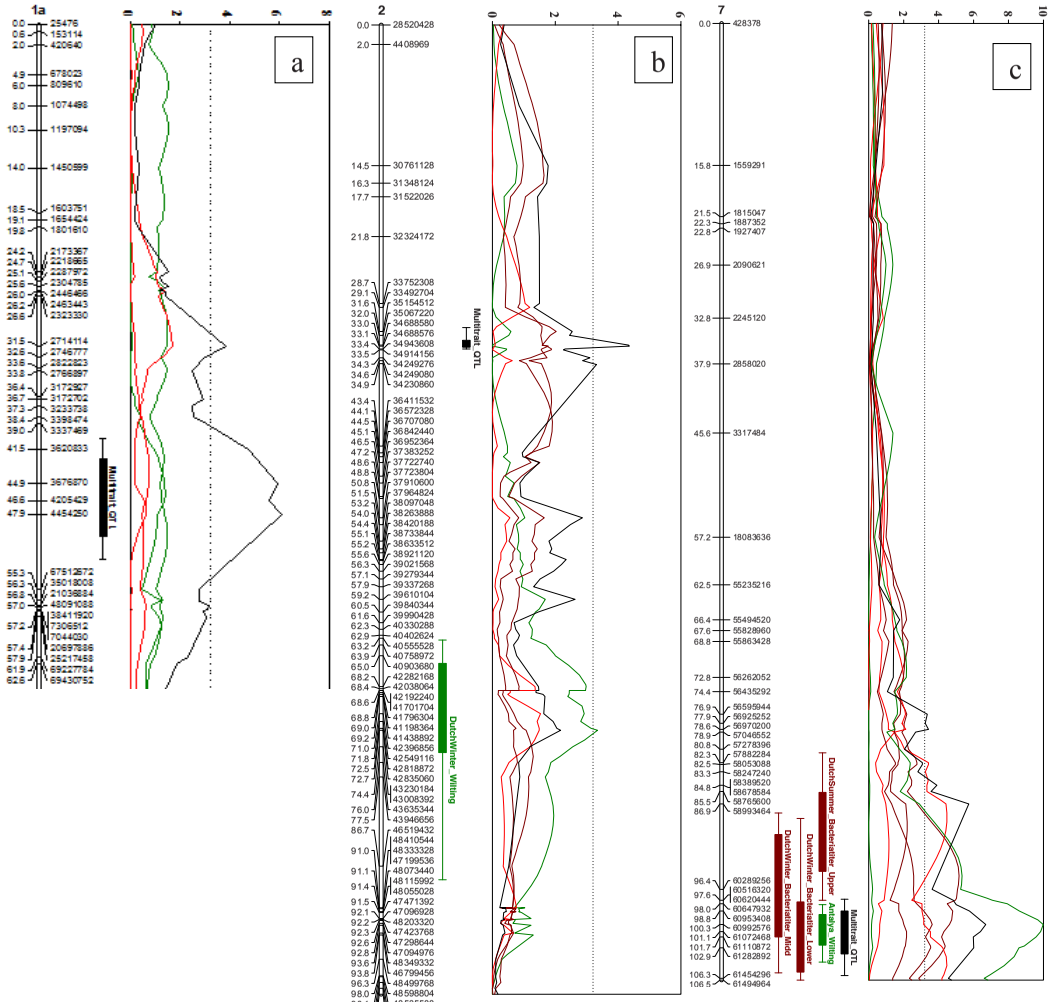
Chromosome	**Number of linkage groups**	**Average cM distance between markers**	**Chromosome**	**Number of linkage groups**	**Average cM distance between markers**
1	2	1.3	7	1	2.7
2	1	1.2	8	2	2.1
3	3	2.2	9	1	1.1
4	1	1.4	10	2	3.0
5	1	1.7	11	1	1.2
6	1	1.3	12	1	1.1

Table 3. Heritability of traits and correlation between traits.

Traits	**Heritability**		**Correlation matrix**										
1 Antalya_Wilting	0.78	1	-										
2 Dutch Summer_Stem discoloration	0.72	2	0.01	-									
3 Dutch Summer_bacteria titer_low	0.78	3	0.11	0.28	-								
4 Dutch Summer_bacteria titer_mid	0.64	4	0.25	0.27	0.31	-							
5 Dutch Summer_bacteria titer_upp	0.43	5	0.12	-0.04	-0.06	0.47	-						
6 Dutch Summer_Wilting	0.87	6	0.05	0.12	0.14	-0.28	-0.43	-					
7 Dutch Winter_Stem discolotation	0.65	7	-0.03	0.21	0.02	0.04	0.06	-0.14	-				
8 Dutch Winter_bacteria titer_low	0.59	8	0.16	0.18	0.09	0.34	0.34	-0.15	0.39	-			
9 Dutch Winter_bacteria titer_mid	0.69	9	0.21	0.19	0.16	0.40	0.43	-0.11	0.36	0.82	-		
10 Dutch Winter_bacteria titer_upp	0.66	10	0.07	0.22	0.05	0.34	0.39	-0.14	0.22	0.64	0.58	-	
11 Dutch Winter_Wilting	0.74	11	0.26	0.47	0.23	0.28	0.14	0.33	0.34	0.44	0.38	0.41	-
			1	2	3	4	5	6	7	8	9	10	11

Table 4. Chromosome, genetic distance, the physical length and explained variance of each detected QTL for three *Cmm* related traits in three different environments by single and multi-trait QTL approaches. Genetic distance and the physical length of each detected QTL were calculated based on 2-LOD QTL interval confidence level.

	Single trait analysis				**Multi-trait analysis**			
Traits	Chromosome	Genetic distance (cM)	Physical length (Mb)	Explained Variance (%)	Chromosome	Genetic distance(cM)	Physical length (Mb)	Explained Variance (%)
Wilting in Dutch winter environment	2	6	1	13.7	1,7 and 8b	14, 11 and 9	3.7, 0.8 and 2.2	9.3, 7.0 and 6.4
Stem Discoloration in Dutch winter environment					1 and 7	14 and 11	17 and 0.8	7.8 and 4.5
Bacterial titer in Dutch winter environment (Lower part)	7	5	1.1	16.3	2 and 7	5 and 11	1 and 0.8	11.7 and 16.3
Bacterial titer in Dutch winter environment (Middle part)	7 and 12	18 and 6	2.4 and 1	21.5 and 12.6	2, 7 and 12	5, 11 and 20	1, 0.8 and 15	9.6, 22.4 and 5.7
Bacterial titer in Dutch winter environment (Upper part)					7	11	0.8	7
Wilting in Dutch summer environment					1 and 8b	14 and 9	17 and 2.2	18.1 and 20.7
Stem discoloration in Dutch summer environment								
Bacterial titer in Dutch summer environment(Lower part)								
Bacterial titer in Dutch summer environment(Middle part)	12	5	0.2	13.3	7 and 12	11 and 20	0.8 and 15	11.9 and 14.1
Bacterial titer in Dutch summer environment (Upper part)	7	12	2.4	18.2	1 and 7	14 and 11	17 and 0.8	5.4 and 17
Wilting in Antalya environment	7	20	2.4	34.9	7	11	0.8	32.5


a
b
c
1a
2
7
Multitrait QTL
DutchWinter_Wilting
DutchWinter_Bacterialblr_Midd
DutchWinter_Bacterialblr_Lower
DutchSummer_Bacterialblr_Upper
DutchSummer_Wilting

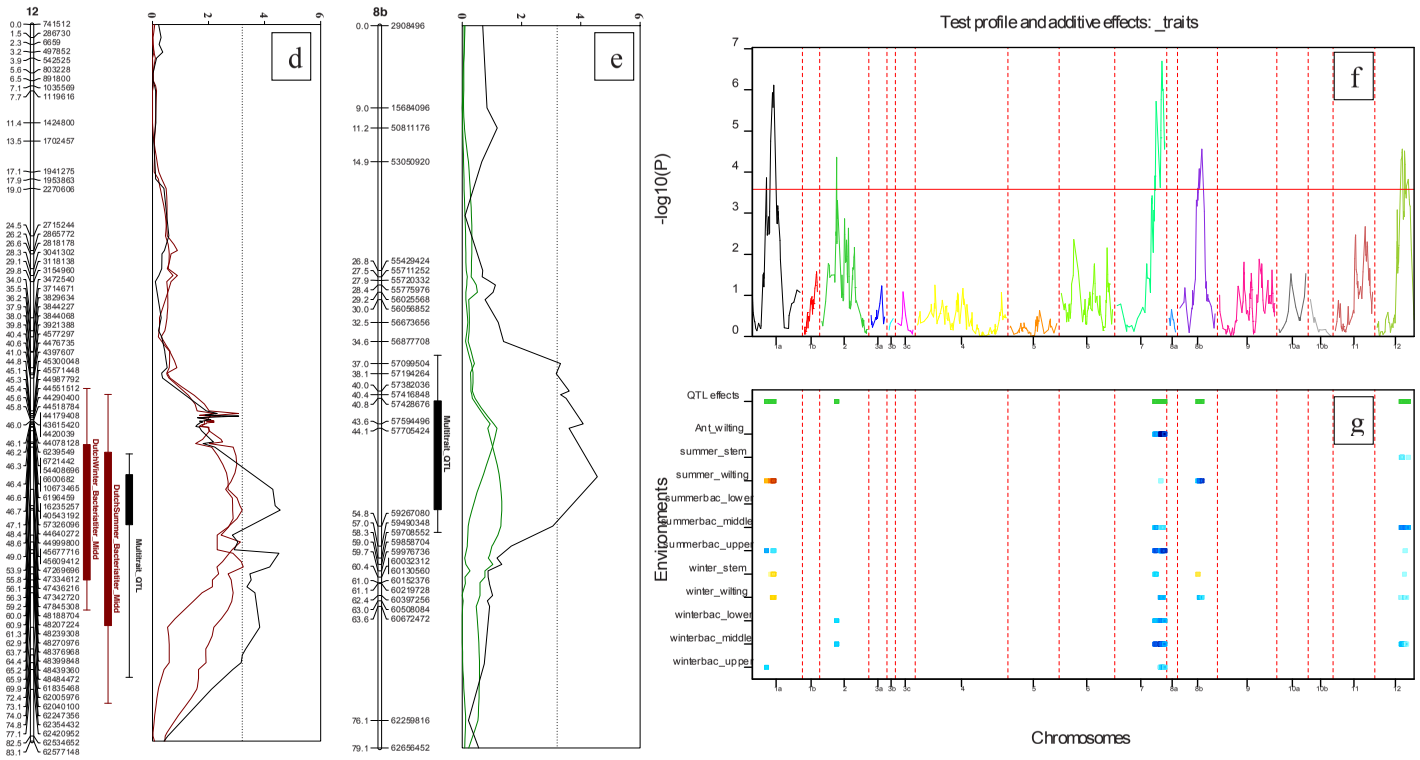


Figure 2: a-e: *QTL profile of single trait approach*. Genetic distance (cM) and physical position of each QTL is shown with number at left side of graph. Horizontal side of the graph showing a LOD score of QTL. Bar showing 1and 2-LOD intervals of each detected QTL and undetected QTL by single approach but detected by multi-trait approach for this trait. Dashed line showing genome-wide threshold. Green color represent 'Wilting' trait, red color represents 'Stem discoloration' trait, brown colour represents 'Bacterial titer' traits and black color showing multi-trait test analysis intervals. f-g:*QTL profile of multi- trait approach*. (f) bar shows statistical power (-log10(p)) and QTL that above genome wide threshold (g) bar shows traits in different environments with blue allele from *S. pimpinellifolium* and red allele from *S. lycopersicum* parent.

Chapter 5

Tools for introducing resistance to *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) in tomato

Tools for introducing resistance to *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) in tomato

Yusuf Sen[1,3], Aylin Kabaş[2], Rudil Bayyurt[1], Richard G.F. Visser[1] and Sjaak van Heusden[1,4]

(1) Wageningen UR Plant Breeding, Wageningen University and Research Centre, PO Box 386, 6700 AJ Wageningen, The Netherlands (2) West Mediterranean Agricultural Research Institute, Demircikara Mh. PaşaKavakları Cad. N: 11, Muratpaşa Antalya, Turkey (3) Graduate School Experimental Plant Sciences, Wageningen Campus, 6807PB Wageningen, The Netherlands. (4) Corresponding author, Email: sjaak.vanheusden@wur.nl.

Abstract

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*) is considered the most serious bacterial threat in tomato and is the causal agent of bacterial canker. Bacterial canker can be transmitted via seed, and outbreaks occur frequently and result in high yield losses in production areas. The organism is worldwide subjected to quarantine regulations and there is no cultivar on the market containing high levels of *Cmm* resistance. We have previously reported *Solanum arcanum* LA2157 as a resistance source and a genetic analysis of an F_2 population revealed three Quantitative Trait Loci. Our aim was to fine map the known QTLs and to start the development of nearly isogenic lines (NILs) containing the QTL regions. To develop nearly isogenic lines embryo rescue was needed and to reduce the number of backcrosses marker assisted background selection was used. On average 1.5% of the donor genome is still present in the BC_3 NILs. We tried to confirm on a high density genetic map the QTL using an F_4/F_5 population, but didn't succeed.

Keywords: Clavibacter, *S. arcanum*, Fine mapping, NIL development, marker assisted background selection, QTL

Introduction

Genetic variation can be qualitative and quantitative. Qualitative characters often have a clear segregation pattern whereas quantitative variation is scored on a continuous scale. Several genes are involved in quantitative traits and there is a strong interaction with the environment. Different statistical methods are available to analyse segregating mapping populations and to pinpoint regions with genes of interest and different mapping studies can be used in tomato for these kinds of studies (F_2, Recombinant Inbred Lines - RIL, Backcross populations - BC_1-$BC_2$and IL populations). These mapping populations are made through the

crossings of two homozygous parents differing for the trait under study. In tomato it is possible to develop an F_2 population within a year. A disadvantages of an F_2 population is that it is virtually impossible to keep such a population through cuttings alive (and disease free) for several years. Tissue culture is too expensive and sometimes unpredictable. Quantitative trait loci detected in a mapping population need to be confirmed in other populations. Such a population can be a RIL population which is made by selfing and single seed descent of a sufficient number of F_2 plants. Making RILs with distantly related species of tomato such as *S. arcanum* is often troublesome and many times it is not possible to go to a substantial large F_6 RIL population via single seed descent. RILs allow the detection of additive effects and epistasis but not of dominance effects since all plants are mainly homozygous. Nearly isogenic lines (NILs) can be used to perform detailed studies in genetically uniform plants in time and space. To speed up the making of NILs, marker assisted background selection can be applied [136]. In some cases homozygous NILs may not be obtained due to lethality and sterility factors [137]. NILs, or a set of sub-NIL population, can be the starting point for fine mapping [138].

Clavibacter michiganensis subsp. michiganensis (*Cmm*) is a gram positive plant bacteria causing bacterial canker in tomato and is considered to be the most harmful bacteria in tomato [3]. Symptoms appear as unilateral wilting and at a later stage the whole plant can wilt and die. Besides wilting stem canker and stem discoloration can be found in infected plants [15]. *Cmm* is a seed transmitted disease and even a few infected seeds (one to five seeds per 10.000 seeds) can result in a serious epidemic in the field [17].There is no cultivar on the market with high levels of *Cmm* resistance. Development of tomato lines with sufficient levels of resistance to *Cmm* can reduce *Cmm* outbreaks in tomato production areas. Previously, we have identified three resistance related QTL using an F_2 population derived from a single F1 hybrid of the cross between *Solanum arcanum* LA2157 and *Solanum lycopersicum* cv. Solentos [73]. Due to the low density genetic map the genetic distances between QTLs and flanking markers were large. The availability of high density marker systems [119] makes it now possible to saturate the map in the QTL regions. Introgression of these QTLs in a *Solanum lycopersicum* background will allow breeders to use this material to obtain tomato cultivars with higher levels of resistance to *Cmm*.

In this study, we have fine mapped earlier reported QTLs using different SNP marker technologies. We have also developed nearly isogenic lines containing those reported *Cmm* resistance QTLs with the help of embryo rescue and marker assisted background selection.

Materials and Methods

Plant material

For fine mapping DNA of 304 F_2 plants (the DNA used were the 20 year old stock solutions of 324 plants from the original F_2 population) was used and if needed DNA was genome wide amplified [139]. New F_2 plants, from the cross between *S. lycopersicum* cv Solentos and *S. arcanum* LA2157 were screened with markers flanking the three QTL regions and selected plants were backcrossed to *Solanum lycopersicum* cv Moneymaker to obtain backcross lines. In 1993-1996, 58 RILs (mix of 16 F_4, 11 F_5, 22F_6 and 9 F_7) were obtained from the same cross by single seed descent. The RILs were in different stages of development and always the last generation of a certain line.

Embryo rescue

Seeds of the backcrosses were excised from immature fruits (20 to 30 days after pollination; DAP) in sterile conditions and were plated on medium. Two kinds of culture mediums were used which consisted of three different concentrations of gelrite. Media 1: MS+GA3(0,35mg/l)+BAP(0,2mg/l)+6% sucrose prepared in 0%, 0.5% and 2% gelrite. Media 2:MS+kinetin(0,2mg/l)+GA3(0,35mg/l)+6% sucrose also prepared in 0%, 0.5 % and 2% gelrite. Plantlets that germinated from this media were acclimatized and transferred to the greenhouse. Genomic DNA was extracted from these plants and genotyped using High Resolution Melting (HRM) [140] and KASPar technology(LGC genomics, England). KASPar is a PCR-based genotyping method combined with allele-specific amplification followed by fluorescence detection. Selected hybrid plants were backcrossed to *S. lycopersicum* cv. Moneymaker to produce the F_2BC_2lines.

Development of markers for fine mapping

For fine mapping, we have used two different SNP genotyping technologies; High Resolution Melting (HRM) and KBioscience Allele Specific PCR (KASP). For HRM, PCR fragments in the target region of a maximum size of 400 basepairs were sequenced and SNPs were identified using LaserGene DNASTAR 9.0 SeqMan software package. After identifying SNPs, primers and probes for the light scanner SNP genotyping were selected using the Idaho technology Light Scanner software. HRM was done as follows; PCR was performed in a volume of 10 µl with each reaction containing 30-40ng DNA template, 1µl PCR buffer solution (10x), 1µldNTP (10µM), 1µlTaq polymerase, 1µl LCgreen, 1 µl forward primer (5µM), 1µl reverse primer (1µM) and 1µl Probe (5µM). Before amplification 15µl of oil was added on the surface of each sample. Amplification conditions were 94˚C for 30 secs followed by 55 cycles (30secs 94˚C, 30 secs 72˚C and for 30 secs Tm) and after the 55 cycle reactions one time 30 secs at 94˚C. Afterwards the melting profiles were analysed according the protocol of Idaho Technology Inc. For the KASPar assay, target PCR fragments were sequenced and SNPs were identified using LaserGene DNASTAR 9.0.A large number of SNPs were determined using a

custom made Infinium bead array originally designed for other purposes [119]. This array was also used for analysis of the RIL population.

Since the tomato sequence is known (The Tomato Genome Consortium, 2012) the positions (release 2.40) of the SNPs were also known. The flanking regions 2 x 75bp of each SNP position were used for primer design of the KASPar assay. SNP markers that were developed for fine mapping in the target regions were also used for NIL development.

Genetic map

A custom-made Infinium Bead array [119] was used for genotyping the 58 RILs. A genetic map was constructed using Joinmap4.1 software [120] using regression algorithm with Kosambi mapping function. We have chosen a LOD score 4 for grouping, if needed this LOD score was raised for specific groups. Identically segregating SNPs were considered as a single marker. Markers with a poor goodness-of-fit in the map were excluded.

Background selection

Of the markers on the Infinium Array a total of 1927 SNPs were scored between *Solanum lycopersicum* cv Solentos and *Solanum arcanum* LA2157. Sixty eight of these were selected (5 to 6 markers per chromosome) based on genomic position (~20 cM apart) and expected recombination frequency.

Disease screening

Inoculation was done at the sixth leaf stage by removing the second leaf with scissors and injecting 5µl of 10^8 cfu/ml in the wound. The inoculum consisted of a mix of fourteen different strains. Plants were kept in pots with soil for 3 months and symptoms of bacterial canker were recorded starting months after inoculation using the following scale: no symptoms, score 1 = 0 to 25% leaf wilting, score 2=25% to 50%,score 3= 50 to 75% and finally score 4= 75% to 100% wilting and the plant is death. Due to germination problems, we were able to use 40 of the 58 RIL for phenotyping and on average 4 plants per line (3 to 13 plants per line). Both parents were represented by 10 plants. Disease screening was done in Antalya in the south of Turkey.

QTL analysis

Single trait analysis of data was done by MapQTL6.0 software[121] using an interval mapping algorithm. The 10.000 times permutation test was applied to determine linkage and genome wide threshold for QTL detection. The logarithm-of-odds (LOD) profiles from interval mapping were inspected and the marker closest to each LOD peak were selected as cofactors then the backward elimination procedure was performed to select significant cofactors. This backward elimination procedure was performed until stable cofactor subsets has been obtained. Remaining cofactor(s) was used for further restricted-MQM mapping

analysis. For determination of QTL intervals we have used 1 and 2-LOD interval of rMQM test.

Estimating heritability

Total genotypic variance was from one-way random effects of analysis of variance using GenStat 14.0 version. Total variance was portioned in two; variations between lines (Vg) and variance within lines (Ve). Broad-sense heritability was (H^2) estimated using both variance of the components according to the formula; H^2 =Vg/(Vg +Ve)

Detection of epistasis

To investigate the allelic effects of QTLs, we utilized two-way analysis of variance (ANOVA) to test the marker closest to the peak of each QTL. The mean phenotypic value was used as a dependent variable and the marker closest to each detected QTL were used as fixed factors. The general linear model module of the statistical package SPSS version 19.0 was used to perform analysis of variance.

Results

For fine mapping of the three QTL regions, we used 20 years old DNA (stored at -20˚C) of the previous F_2 population. The aim was to use the progress in mapping technologies for adding more markers to the QTL regions of the Restriction Fragment Length Polymorphism (RFLP) map of 51 markers. This should lead to a more precise location of the three QTLs. In total 40 SNP markers were chosen of which 12 didn't work. The DNA of in total 277 of the 324 individuals could be used for fine mapping. Our previous results showed a putative QTL on Chromosome 5 of which no QTL interval could be calculated (Fig.1a). Fine mapping increased statistical detection power and promoted the putative QTL on Chromosome 5 to a real QTL with a LOD score above the genome wide threshold (Fig.1b). The QTL region of QTL5 (LOD 1 interval) was reduced to ~1 cM which corresponds to 28 Mb. Fine mapping also improved the robustness of the QTL on Chromosome 7. The QTL7 interval was located between marker TG418 and TG61 (genetic distance 30 cM; physical length 3.5 Mb) (Fig.1c), after fine mapping the LOD-1 interval of the QTL was reduced to the region between marker TG418 and the marker on position 1405593 corresponding to a genetic distance of 13.6 cM and a physical length 1.2Mb (Fig.1d). For QTL9, the fine mapping didn't reduce the size of the interval, this remained between marker TG254-TG223 with 30 cM and0.5 Mb (Fig.1e,f). These studies clearly confirm that our analysis in 1999 was good and that indeed three QTLs are present. This paved the way for the following step: the development of nearly isogenic lines. The originally genotyped and phenotyped F_2 plants were for obvious reasons not available anymore but fortunately F2 seeds were. We started with analyzing new 51 F_2 plants and used flanking markers of the QTL regions, 19 F_2 plants were selected containing the

three QTLs homozygous for *S. arcanum*. With these plants 874 backcrosses on *S. lycopersicum* were done to get enough F_2BC_1 fruits. However, only 389 crosses resulted in fruits of which we were able to rescue 50 embryos. Thirty two of the 50 rescued embryos started to grow and finally we obtained 12 healthy F_2BC_1 plants after acclimatization. Those 12 F_2BC_1 plants were genotyped to confirm the presence of the QTL region. Using two SNP genotyping technologies, we confirmed that four of the lines contained one or more of the three QTL regions heterozygous. The remaining ones were selfed cv Moneymaker. The four F_2BC_1 plants were backcrossed with cv Moneymaker once again. One of those four plants didn't give seeds which prompted us to do an additional embryo rescue for this genotype. The other three lines gave F_2BC_2 progenies. Using KASPar technology, we showed that 27 F_2BC_2 plants from the three BC_1plants were still containing the QTL region in a heterozygous state. In order to get F_2BC_3 plants, F_2BC_2 plants were backcrossed and after marker selection 224 BC_3 were found with one of the QTLs heterozygous present. About 45% of the backcrosses were successful and the germination rate was about 64%.

For the embryo rescue we have used two different media and three treatments. In our study, all treatments and media have been found successful for germination. However, we didn't have a sufficient number of germinated seeds to compare media and treatments in a significantly sound way. We do believe however that the medium containing gibberillic acid (GA) with solid gelrite (2%) was the most effective combination for tomato embryo rescue. After obtaining 224 F_2BC_3 plants, the next step was the selection of plants with one of the QTL(s) with the lowest percentage of the donor genome. To determine this we have used 68 SNP markers covering the genome of tomato (marker assisted background selection). Four markers didn't give an amplification resulting in a total of 64 informative markers. The percentage *S. arcanum* varied between 1.5 to 24.2 %. We were able to select 20 plants with a percentage from 1.5 to 6.6 % and containing one of the QTLs. With these 20 plants we will do an additional backcross and collect seeds after selfing. The high allele proportion of the donor genome in some non-selected regions was striking, a chromosomal region below the centromere on Chromosome 2 (region around 28,5 Mb) was present in 50-70% and the physical region between 6,4 Mb and 46,7 Mb area on Chromosome 11 (8 cM) was present in 20-45% of the BC_3plants. Another example of high allele proportion was that plants selected for QTL5 had 65% of *S. arcanum* genome on Chromosome 8 in the region between 2.9 and 26.7 Mb (12 cM). These regions were also distorted segregating in the RIL population (data not shown) discussed below.

Our laboratory has done an effort in the nineties to obtain RIL populations of a number of wild relatives of tomato (*S. arcanum* LA2157, *S. pimpinellifolium* G1.1554, *S. habrochaites* LA1777 and *S. pennellii* LA716). This was only successful for *S. pimpinellifolium* (Chapter 4 of this thesis) for the other three combinations in every round of selfings the potential size of the F_6 RIL population became smaller and smaller due to plants without flowers and/or non-germinating seeds. This all resulted in small F_6 mapping populations. For our studies we saw a possibility to use a combination of F_4, F_5, F_6 and even F_7 lines of the *S. lycopersicum/S.*

arcanum combination; in total 58 lines of which only 40 had viable seeds. In our laboratory there was also a custom made SNP array available [119] and of the 5528 SNPs on this array with known positions of the tomato sequence 1927 SNP markers were polymorphic between *Solanum lycopersicum* cv Solentos and *Solanum arcanum* LA2157. Finally 700 SNP markers (excluding all but one of identically segregating loci) were used to make a genetic map. The total genetic map size was 927 cM consisting of 22 linkage groups. Due to a lack of markers in regions with a high recombination, some chromosomes are represented by more than one linkage group. Average genetic distance per marker was 1.3 cM and there were 60 markers per chromosome on average. The largest gap was detected on Chromosome 3 (17 cM/11.4 Mb).We have performed QTL analysis in this RIL population for the confirmation of previously detected QTLs. We have detected one major QTL for wilting on Chromosome 6 with an explained variance of 32% in a 5 cM/1.2 Mb region (Fig.2B). We also detected two putative QTLs for wilting just below the set genome wide threshold on Chromosome 9 with an explained variance of 17.3% and on Chromosome 11 with an explained variance of 13.1%. The heritability of wilting was 0.80. No significant interactions between major QTL and putative QTLs were found and the different QTLs are additive. Considering the major QTL on Chromosome 6 and the two putative QTLs on Chromosomes 9 and 11, we were able to explain 62% of phenotypic variance which corresponds to 77% of genetic variance. On a scale of zero to four a plant with a combination of QTL6 + QTL9 gives a disease score of 0.4, QTL6 + QTL11 and QTL9 + QTL11 give a disease score of 0.8, all three QTL together results in a score of 0.3.

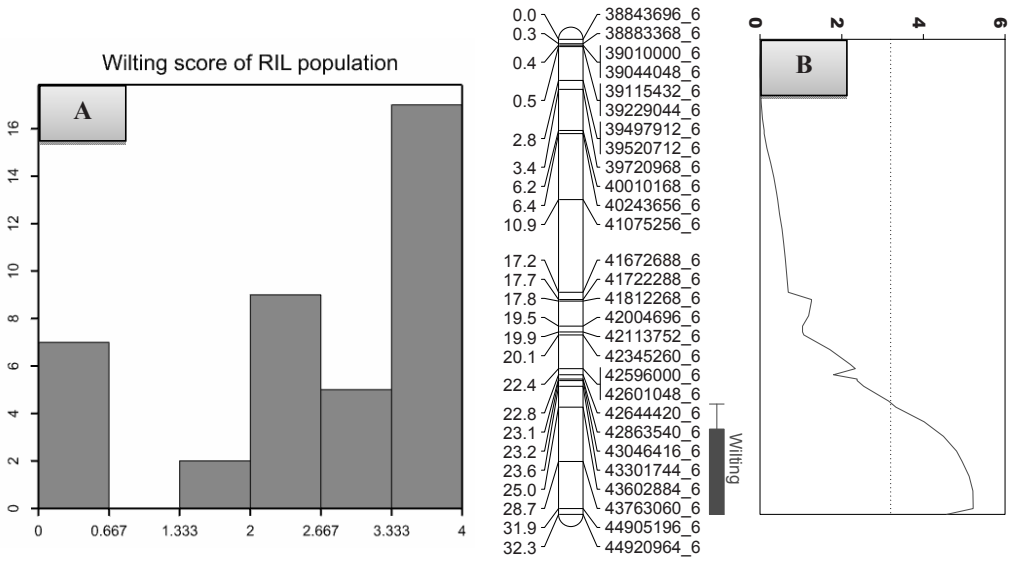


Figure 2. 2A*:* Distribution of wilting of the 40 recombinant inbred lines derived between *Solanum lycopersicum* cv. Solentos and *Solanum arcanum* LA2157. X-axis shows disease score ranging from on a scale from 0 to 4 and Y-axis shows the number of lines per class. 2B: The profile of wilting for major QTL on Chromosome 6. The dashed line indicates the genome-wide threshold for QTL determination. Genetic distance (cM) and physical position (bp) of the QTL is shown at left side of graph. Horizontal side of the graph shows the LOD score. The bars show the 1and 2-LOD interval.

Discussion

Fine mapping in an F_2 population can be achieved by increasing the population size (more recombinations), and/or increasing marker density. Restriction fragment length polymorphisms are very laborious and that was the reason that in the previous study [73] marker density in the genetic map was very low, on average 4.2 markers per chromosome, the QTL regions were on average 20 cM to 50 cM. We choose to add more markers in the QTL regions, made possible due to recent developments. Adding more markers makes it possible to reduce the size of QTL intervals. The region of QTL5 and QTL9 could be determined more precisely but it was still considerably large because the population size was not increased. In other words the recombination frequency becomes the bottleneck. An F_2 population with many markers and a population size of 500, will not reduce the size of the QTL region to less than 10 cM [141]. A good and reliable phenotyping is also of utmost importance, not optimal phenotyping makes it difficult, especially for QTLs with small effects, to minimize QTL regions. The QTL on Chromosome 7 had a large effect and the region harboring this QTL could be reduced. Using SNP markers for fine mapping in

combination with the tomato sequence made it possible to obtain the physical position of the QTL regions. Although DNA of the F_2 population was isolated long ago in 1993, we were able to make use of the 20 years old DNA samples for SNP genotyping.

Nearly isogenic lines make it possible to study resistance mechanisms in a *S. lycopersicum* background thoroughly. NILs can also be the starting point for further fine mapping and for introducing the traits via introgression breeding (no embryo rescue needed anymore).In making the hybrid and later in the first backcrosses embryo rescue was needed. Theoretically F_2BC_4 and F_2BC_5 lines possess 3.1 and 1.5% donor genome respectively. In tomato 2-3 generations are possible per year and in each generation marker selection is required in order to maintain the donor QTLs. Using marker assisted background selection, we were able to obtain F_2BC_3 lines with only 1.5% of the donor genome which is equivalent to BC_5 generation. We cannot exclude that through double recombination small donor regions, between the markers we used, are present. Surprisingly some chromosomal regions remained preferably like the donor. Examples are a chromosomal region below the centromere on Chromosome 2 and the top part of Chromosome 8. An explanation might be that these regions influence fitness.

We constructed a genetic map using 1790 potential SNPs. Due to high polymorphism rate between the two species, we were able to construct a high density genetic map even with the small sized RIL population (40lines). The dense genetic map was helpful to increase the resolution for detection of QTLs. Confirmation of QTLs in another population is necessary to be sure it was not a false positive QTL [138, 142]. We have tried to confirm the previously published QTLs [73] using the RIL population of 40 lines. We didn't detect any of the known QTLs in the RIL population but identified some new QTL(s). A major QTL on Chromosome 6 and two putative QTLs on chromosomes 9 and 11. A combination the major QTL and either of the putative QTLs gives a similar resistance level as the resistant parent. The small sized population in our experiment can be the reason that QTL(s) are missed but might also cause an overestimation of the number of QTL(s) effects which is known as the Beavis effect [143]. Screening of experiment 1 (the Netherlands) and 2 (in Turkey) were done in soil pots, but under quite different environmental conditions. Furthermore in experiment 1,one aggressive strain was used and in experiment 2 a mix of fourteen different aggressive strains was used. There might be an effect of *Cmm* strains which indicates gene-for-gene interactions but gene-for-gene models have not been reported for *Cmm*-tomato interactions [86]. Another explanation might be differences in quantity of cell wall degrading enzymes between strains. The phenotyping was also done in a somewhat different way, in experiment 1 a non-quantitative scale was deployed and plants were cut at a 6th leaf stage after inoculation which doesn't allow observations during plant development.

In conclusion, *S. arcanum* LA2157 is a very good source for *Cmm* resistance due to its performance under different environment conditions and its performance with different *Cmm* strains. Embryo rescue was needed to make use of this source for breeding purposes.

Mapping of the 3 previously identified QTLs was improved without increasing the population size. A set of F_2BC_3 lines (NILs) contain relatively a low percentage of the genome of *S. arcanum*. These NILs and combinations of these NILs (QTL5/QTL7 in one line) will make more extensive studies possible under different conditions and with different strains of the pathogen. These studies about genotype-strain interactions (gene-for-gene interaction) and/or strong environment x QTL interactions will hopefully explain why in our RIL population we couldn't confirm the QTLs but instead identified at least one new QTL. Using the *S. Arcanum* source of *Cmm* resistance (although there are still bacteria) we aim at providing the tools to develop *Cmm* resistant commercial cultivars that will prevent devastating outbreaks and complete losses of the crops. This will be very beneficial to growers in many parts of the world.

Acknowledgements:

We are thankful to Dr. Geert Jan de Klerk for helpful advises about embryo rescue medium selection and Bernadette van Kronenburg-van de Ven from for technical help (Wageningen UR Plant Breeding, Wageningen University and Research Centre).

Table 1: Number of phenotyped RIL lines with previous and new QTL regions.

QTLs	***S. lycopersicum* allele**	***S. arcanum* allele**	**Heterozygous**
Previous QTL5	27	13	
Previous QTL7	11	27	2
Previous QTL9	21	13	6
Previous QTL5+ Previous QTL7	4	6	
Previous QTL5+ Previous QTL9	15	5	
Previous QTL7+Previous QTL9	6	9	1
NewQTL6	21	15	4
NewQTL9	19	16	5
New QTL11	20	15	5
New QTL6+New QTL9	12	6	2
New QTL6+ New QTL11	10	6	2
New QTL9+New QTL11	9	6	1

.

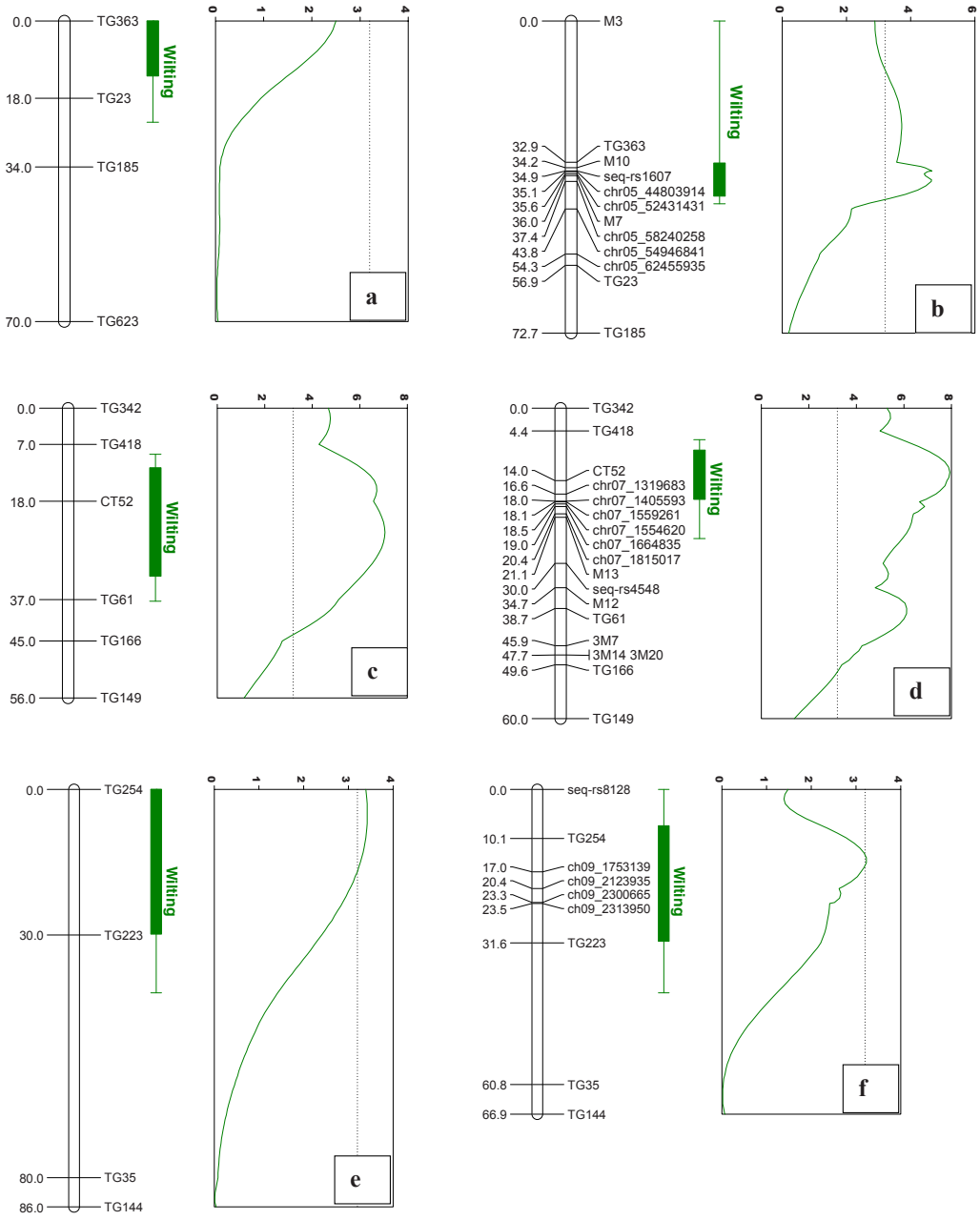


Figure 1. The profile of 3 QTLs, on Chromosome 5 before (a) and after (b) fine mapping, on chromosome 7 before (c) and after (d) fine mapping and on chromosome 9 before (e) and after (f) fine mapping. Dashed line indicates genome-wide threshold for QTL determination. Genetic distance (cM) and physical position of each QTL is shown with number at left side of graph. Horizontal side of the graph showing a LOD score of QTLs. Bars show 1 and 2-LOD intervals.

Chapter 6

Multilocus Sequence Typing Analysis of *Clavibacter michiganensis* subsp*. michiganensis* (*Cmm*) strains in Turkey

Submitted for publication

Multilocus Sequence Typing analysis of *Clavibacter michiganensis subsp. michiganensis* (*Cmm*) strains in Turkey

Yusuf Sen[1,5], Yesim Aysan[2], Duygu Ozdemir[1], Fien Meijer-Dekens[1], Mustafa Mirik[3], Jan van der Wolf [4], Richard G.F. Visser[1] and Sjaak van Heusden[1,6]

(1) Wageningen UR Plant Breeding, Wageningen University and Research Centre, PO Box 386, 6700 AJ Wageningen, The Netherlands (2) Çukurova University, Faculty of Agriculture, Department of Plant Protection, 01330 Adana, Turkey (3) Namik Kemal University, Department of Plant Protection, TR-59030 Tekirdag, Turkey (4) Plant Research International, Biointeractions and Plant Health, Droevendaalsesteeg 1, 6708 PB, Wageningen, The Netherlands (5) Graduate School Experimental Plant Sciences (6) Corresponding author, Email: sjaak.vanheusden@wur.nl

Abstract

Clavibacter michiganensis subsp. michiganensis (*Cmm*) is a gram positive plant bacterium and is considered to be the most harmful bacterium in tomato. We have carried out a study on 108 new *Cmm* strains that were collected between 1996 and 2012 in different parts of Turkey. Multilocus sequence typing (MLST), based on five housekeeping genes and three virulence related genes was done to determine the diversifying mechanism and the degree of clonality. The population structure of the collection was assessed, a split network analysis was visualized and a phylogenetic tree based on this collection was constructed. The relation of our collection of strains with other *Cmm* strains was assessed.

Introduction

Clavibacter michiganensis subsp. michiganensis (*Cmm*) is a gram-positive plant bacterium belonging to the Actinobacteria and is the causal agent of bacterial canker in tomato. It is one of the most important plant pathogenic bacteria [14] and is considered to be the most harmful bacterium in tomato [3]. *Cmm* is a quarantine organism in European Union and some other countries [8, 108]. Although it mainly causes damage in tomato, pepper and eggplant are also recognized as hosts [8]. The bacteria can be transmitted via seed and theoretically even a few infected seeds (one to five seeds per 10,000) can result in an epidemic outbreak in the field [17] resulting in serious yield losses[12]. Disease outbreaks in new area's often are due to the use of infected seeds, but the use of infected planting

material may also be the source of initial infections. Bacteria can reside in soil or in crop debris in or on soil till the next season and be a source of new infections.

Cmm is genetically and phenotypically a diverse subspecies [8]. The various bacterial haplotypes (Sequence Types) can differ in virulence and in their ability to spread in the ecosystem [146]. Studies on population structures have been helpful to understand introduction pathways and indirectly it has answered questions on how *Cmm* evolves. The information has also been used for the selection of representative panels of strains in studies on *Cmm*.

In recent study on population structures of *Cmm*, multilocus sequence typing (MLST) was used, to determine isolate structure [146]. MLST is based on allelic variation within genes between strains. Since mutation accumulation in housekeeping genes is relatively slow it is a good tool to study genetic relations of strains collected globally and not only in specific regions [147]. Typically, a MLST phylogenetic analysis is based on 6 to 10 genes [148]. MLST analysis often is done on the same set of genes which allows data exchange and is suitable for epidemiological studies [149]. For *Cmm*, classifications can be based on differences in virulence and based on a MLST analysis of housekeeping genes [12, 13, 150]. However, it is not clear which genes are most suitable to study the degree of clonality and the relation of them with the virulence level.

The aim of this study was to establish MLST profiles of strains in which the degree of clonality is measured and to construct a phylogenetic tree which shows the relationships between clonal complexes. In addition, a network was drawn between virulent strains, strains with reduced virulence and non-tomato host strains. This makes it possible to predict the virulence level of unknown strain(s) on tomato. In our study, clonal complex analysis (degree of clonality) of 108 new strains was established and a Maximum Likelihood tree of all genes was constructed. A split network analysis between strains was built and the relation of strains based on virulence level and host was determined. In addition, type of forces which play a role in diversifying of our collection were determined.

Materials and Methods

Bacterial strains

108 bacterial strains were collected after disease outbreaks between 1996 and 2012 in different fields and different parts of Turkey. For the phylogenetic analysis, we also included eighteen external strains hereafter will be called “Global Strains”. Global strains were three strains from the Wageningen UR collection, four strains from Israel [11] and fourteen Serbian strains (2 strains from each group, Table 1) [13].

DNA isolation

DNA isolation of bacterial strains was done using Qiagen QIAamp DNA Mini Kit with gram positive bacterial genomic DNA isolation method. Quality and quantity of DNA were inspected by Nanodrop spectrophotometer analysis and agarose gel electrophoresis.

Strain identification tests

Strains were characterized using a stem-inoculation test on tomato , a tomato cotyledon leaf test, Gram staining, an oxidase test, a hyper sensitivity (HR) test on non-host plants, an ELISA test and a PCR with *Cmm*-specific primers.

For the pathogenicity tests, three replicates of young tomato plantlets (*Solanum lycopersicum* Mill cv. H2274) with 3-5 true leaves were inoculated by injection of the stem with a sterile needle with 100 µl bacterial suspension (10^8cfu/ml) of each *Cmm* isolate. Sterile distilled water was used as negative control. After inoculation, tomato plants were covered with clear polyethylene bags for 24 h at 25^{o}C. The bags were removed and plants were moved to a controlled climate room, at 25^{o}C, and 70% RH and a light regime of 16h light and 8h night. Disease development was evaluated 8-10 days after inoculation and re-isolations were carried out with diseased material.

For the tomato cotyledon leaf tests, three replicates of four days old tomato plantlets (*Solanum lycopersicum* Mill cv. H2274) with 3-5 true leaves were inoculated by injection of the cotyledon leaves with the tip of a cotton swab dipped in the bacterial suspension (10^8cfu/ml) of each *Cmm* isolate. After inoculation, the tomato plantlets were incubated in a controlled climate room at 26^{o}C, 60-70% RH and 16h/8h day/night. Disease development was evaluated 3-4 days after inoculation.

The Gram reaction [151], oxidase reaction and hypersensitivity on tobacco leaves [152] and hypersensitivity on *Mirabilis jala* leaves were replicated three times.

For serological tests, *Cmm*-specific monoclonal antibody (BRA 44001 - Agdia) was used to confirm the identity of *Cmm* strains at the species level. Serological identification of *Cmm* strains was performed according to the previously described indirect ELISA method [153].

The PCR assays was performed using *Cmm*-specific primers *Cmm*5-*Cmm*6 according to the described procedure [37].

Gene selection

The sequences of internal fragments of five housekeeping genes were determined, namely of *BipA* encoding GTP-binding protein typA/bipA-like protein*, GyrB* encoding the DNA gyrase

subunit B*, KdpA* encoding the Kpotassium-transporting ATPase subunit A*, LigA* encoding the NAD-dependent DNA ligase*, SdhA* encoding the succinate dehydrogenase flavoprotein subunit[13] and three virulence related genes namely *PpaA* encoding putative extracellular serine protease, *ChpC* encoding serine protease and *TomA* encoding tomatinase, endo-1,4-beta-glycosidase [11] were sequenced of each strain.

Statistical analysis of MLST data

Chromatograms were analysed with LASERGENE DNAStar SeqMan Pro version (DNAStar Inc.). Mega5 [154] was used to align sequences using the ClustalW algorithm and then data were further manually edited. The border of sequences was trimmed according to the coding region for each gene using the sequence of the reference strain hereafter called "Reference" *Clavibacter michiganensis subsp. michiganensis* NCPPB382 (NCBI database). As an outgroup, *Clavibacter michiganensis* subsp. *sepedonicus* (*Cms*) sequence data was used. In addition, sequences of *Clavibacter michiganensis subsp. michiganensis* NCPPB2979 and *Clavibacter michiganensis subsp. nebraskensis* (*Cmn*) were added.

GC content, total number of segregating sites (DNA sites that are polymorphic), nucleotide diversity, number of haplotypes, haplotype diversity and minimum number of recombination events were calculated using DnaSP 5.10 version [155]. The synonymous/nonsynonymous ratio was calculated in this program with the Tajima D model [156]. Three different tests; Tajima D, Fu and Li's D and F outgroup tests, have been performed to measure diversity of the genes. For estimation of the population diversification mechanisms, the nucleotide diversity was plotted versus the haplotype diversity across all loci.

To determine unique sequence types (STs) in the population, each nucleotide difference between isolates within the same gene was considered a different allele and the combination of alleles in the same isolate is assigned as a ST. STs were named according to strain number. Clonal complex structure analysis and group assignment of STs were done using the eBURST v3 program [157]. eBURST analysis is used to detect single locus variants (SLV) and double locus variants (DLV). Bootstrap analysis was run to support ancestral and subgroup founder ST in a clonal complex. eBURST grouping is used to identify groups of related STs where all members assigned to the same group share identical alleles for at least 6 of the 8 loci with at least one other member of the group. Polymorphisms between a clonal complex founder and its single locus variants (SLV) were checked, if the difference was one nucleotide it was assigned as point mutation, if the difference was more than one nucleotide then it was assigned as a recombination event.

Maximum likelihood analysis was done with RAxML 8.0 [158] using the CIPRES gateway platform [159]. Using Mesquite 2.74 [160], concatenated data of genes were obtained. Then the data were transformed to the PHYLIP format for analysis of Maximum Likelihood. Phylogenetic analysis was performed for each individual gene, concatenated housekeeping genes, concatenated virulence related genes and concatenated data of all genes.

Concatenated gene data sets were handled in a gene partition concept. Tree visualizing was done using the FigTree v1.4.0 program.

Split network analysis was carried out by SplitTree 4.9 program [161] using neighbor-net analysis with the Jukes Cantor distance correction method.

Population structure analysis was investigated using a Bayesian-model based clustering approach implemented in the software STRUCTURE 2.3.4 program [162]. The program was run using an admixture model with a burn-in period of 30,000 iterations, followed by 300,000 Markov Chain Monte Carlo (MCMC) repeats. The optimal number of populations (K) was set 1 to 10 with 10 replications for each K. The LOCPRIOR model [163] was implemented and strains were grouped according to year and location where both overlap and this grouping was used as prior for structure analysis. The final number of subgroups was assessed according the L(K) and Δ(K), an ad hoc quantity related to the second order rate of change of the log probability of data with respect to the number of clusters, methods [164].

Results

Strains were collected between 1996 and 2012 in different parts of Turkey. Each strain was isolated from a different field. All strains were isolated from diseased tomato plants except strain number 46 which was isolated from eggplant. All strains were pathogenic on tomato after inoculation of stem or cotyledon, showed a HR response on *Mirabilis japala*, were positive in the ELISA using *Cmm*-specific monoclonal antibodies, were oxidase negative, Gram-positive and positive in PCR using *Cmm*5-*Cmm*6 primers. These tests confirmed the identity of the *Cmm* strains.

All genes that were used in this study are located on the genome of *Cmm* in which disease related genes (*Chp*C, *PpaA* and *Tom*A) are located on the PAI (pathogenicity) island [2]. Amplified fragments of the eight genes were obtained for all isolates but there were some missing values (in total 59 data points). Most missing data points were from housekeeping genes (83%) and only few from disease related genes (17%). The missing values were treated as missing characters in phylogenetic analysis. The size of the concatenated sequence of eight genes was 4472 bp corresponding 0.0015 % of genome (3.3 Mb).

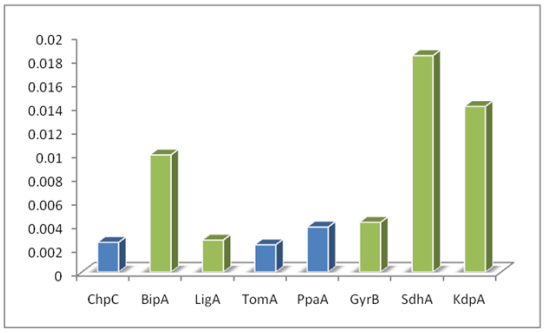


Figure 1: Nucleotide diversity (Y-axis) versus haplotype diversity of eight genes (X-axis). Virulence related genes are in blue color and housekeeping genes are in green color.

Polymorphisms were found in all genes with the lowest values in *Tom*A and the highest in *Sdh*A. The number of alleles ranged from 9 to 19 per gene. One hundred sixty one variable sites were detected of which 35 were parsimony informative (Table 1). Recombination was detected within most of the loci except for *Tom*A and *PpaA*. The GC content, recombination, nucleotide diversity and haplotype diversity were overall higher for the housekeeping genes in comparison with the disease related genes (Table 2). The trend between haplotype diversity and nucleotide diversity, an indicative of evolutionary mechanism, showed an uneven positive correlation (Fig.1). Three independent tests showed a negative value genome wide and for most of the individual genes (Table 1). Ka\Ks ratio, the nonsynonymous-to-synonymous substitution ratio test, was higher than one for *PpaA* and higher ratios were also observed for *Chp*C and *Tom*A. The Ka\Ks ratio was relatively low for the housekeeping genes, except for *Kdp*A (Table 2). Among 108 strains, 3 strains had missing data in 4 genes or more. Forty-two strains were found to be identical to others. All but one of the identical strains as well as strains with a high number of missing values were excluded from further analysis.

Among 108 strains, 63 unique STs were identified. An eBURST analysis with 63 Turkish strains and 18 global strains resulted in two major and 6 minor clonal complexes (Fig. 2a). Of 108 Turkish *Cmm* strains and 18 global strains, ST4 was the biggest clonal complex consisting of 11 STs and representing 23 strains. The other major clonal complex was ST68 representing 13 strains. Minor clonal complexes were ST70-76, ST20–22, ST8-24-28, ST13-15 and ST Israel_402-*Cmm*3356 which represented 8, 6, 9, 7 and 2 strains respectively. Forty-nine singletons were detected in the ST complex analysis. When we consider the year and location of the collected strains only ST13-15, ST87-88 and ST70-76 were homogeneous whereas other STs were heterogeneous (Table 2). eBURST grouping resulted in six groups with 22 singletons. Single locus variants (SLV) and double locus variants (DLV) relation within and between complexes in eBURST groups are shown (Fig. 2b). Strains P10,P501 and P137 from the Serbian collection which had a reduced virulence level behaved as singletons whereas they grouped together in eBURST grouping with other strains. Allelic difference between clonal complex founders and their satellites (SLVs and DLVs) were inspected. At least 14 recombination events and 10 mutation events were detected.

Maximum likelihood for separate genes resulted in a partly incongruent phylogenetic tree (data not shown). There was a partly congruent phylogenetic signal in all genes considering the clonal complex as a unit. But some clonal complexes especially ST68 or ST4 were not visible in some gene trees (examples are *Chp*C and *Lig*A). The phylogenetic tree *Kdp*A and *Sdh*A had a stronger phylogenetic signal than those based on other genes when we consider the amount of visible clonal complexes and the separation from the non-tomato host group.

The phylogenetic tree of the disease related genes (*Chp*C, *Tom*A and *PpaA*) gave also strong phylogenetic signals. All clonal complexes were visible in this tree. But the stronger phylogenetic signals were obtained in the concatenated data of housekeeping genes. All non-tomato host strains were separated with a very high bootstrap value. This tree was very similar to the tree based on all concatenated genes.

Due to a partly congruence of individual gene trees, a final tree was constructed with data of all loci using the maximum likelihood algorithm (Fig. 4). The Maximum Likelihood tree of the concatenated eight genes separated the non-tomato *Clavibacter michiganensis subspecies* (*Cms*, *Cmn* and strain 46) from the rest. Surprisingly strain number 92 and the identical strain 95 were also separated from the other strains. Statistical support (bootstrap value) for the non-tomato host group and strain number 95 was 100% whereas the bootstrap value for the other clades was low (below 50). Major and minor clonal complexes (Fig. 2a) that were detected by eBURST were visible at the edge of lineages (shown as colored groups, Fig. 4). Four Serbian strains (P121, P123, P520 and P521) formed two groups (group 1 and 4) in the original study and grouped with *Cmm*3517 (a Wageningen strain) and NCPPB2979. Other strains from Serbia were grouped as it has been described previously [13] and groups were spread out within groups of Turkish strains. Three strains (P10, P501 and P137) from Serbia representing two groups with a reduced virulence level grouped together in the ML tree. Two strains from Israel (46 and 402), were grouped with two Wageningen strains (*Cmm* 542 and *Cmm* 3356) but other strains from Israel were related with Turkish strains.

Structure software identified two major structures of our *Cmm* strains with global strains based on L(K) and Δ(K) methods in which Cmn and Cms represent one group and the other strains represented the second group (data not shown). Interestingly, ST68 and ST 8-24-28 clonal complexes were structured in different subpopulations when we considered another subpopulation number such as k= 4 or 7.

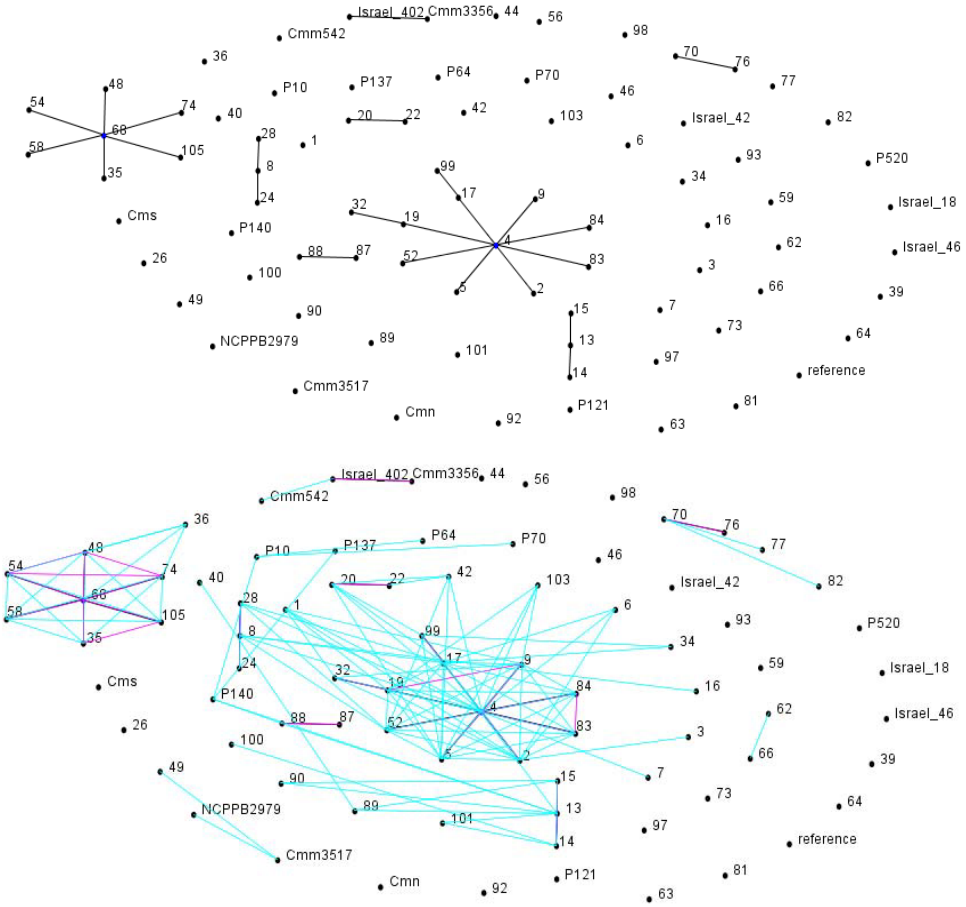


Figure 2 : Clonal complex analysis of 63 Turkish and 18 global strains (a) eBURST grouping of 81 strains(b) Pink lines are SLVs within clonal complex and blue lines are indicating DLV relation between and within STs.

Table 1. Sequence variation and neutrality tests

Gene	FS(bp)	GC (%)	NH	HD	MR	ND	K	Tajima's D	Fu and Li's D	Fu and Li's F
TomA	471	61	9	0.625	0	0.0023	1.067	-2.395**	-6.166**	-5.718**
ChpC	564	53	11	0.325	1	0.0025	1.402	-2.633***	-5.894**	-5.587**
PpaA	519	58	11	0.763	0	0.0038	1.504	-2.146**	-4.441**	-4.321**
LigA	468	72	9	0.587	2	0.0027	1.266	-1.916*	-3.751**	-3.660**
BipA	633	70	11	0.497	1	0.0099	6.286	-0.274	0.948	-0.805
SdhA	658	69	14	0.794	3	0.0183	12.027	1.674	1.294	1.727
KdpA	598	68	12	0.821	7	0.0140	8.355	-1.000	-0.806	-1.073
GyrB	561	69	19	0.772	3	0.0042	2.369	-1.959*	-3.456**	-3.478**
All Loci	4472	64	35	0.928	11	0.0065	10.325	-2.137*	-3.410**	-3.275**

FS: Fragment size, GC: GC content, NH: number of haplotypes, HD: Haplotype diversity, MR: Minimum recombination events, ND: Nucleotide diversity, K: Average number of nucleotide differences and ***($p<0.001$), **($p<0.02$), *($p<0.05$).

Table 2. Polymorphisms of all eight genes

Gene	Total Segregating Sites	Parsimony informative sites	Synonymous changes	NonSynonymous changes	Ka/Ks ratio
TomA	16	2	8	9	0.160
ChpC	31	6	9	13	0.121
PpaA	21	5	5	14	2.684
LigA	15	11	2	14	0.099
BipA	31	23	21	11	0.060
SdhA	34	32	33	1	0.038
KdpA	53	21	29	22	0.350
GyrB	24	11	7	9	0.042
All Loci	161	35	52	91	0.160

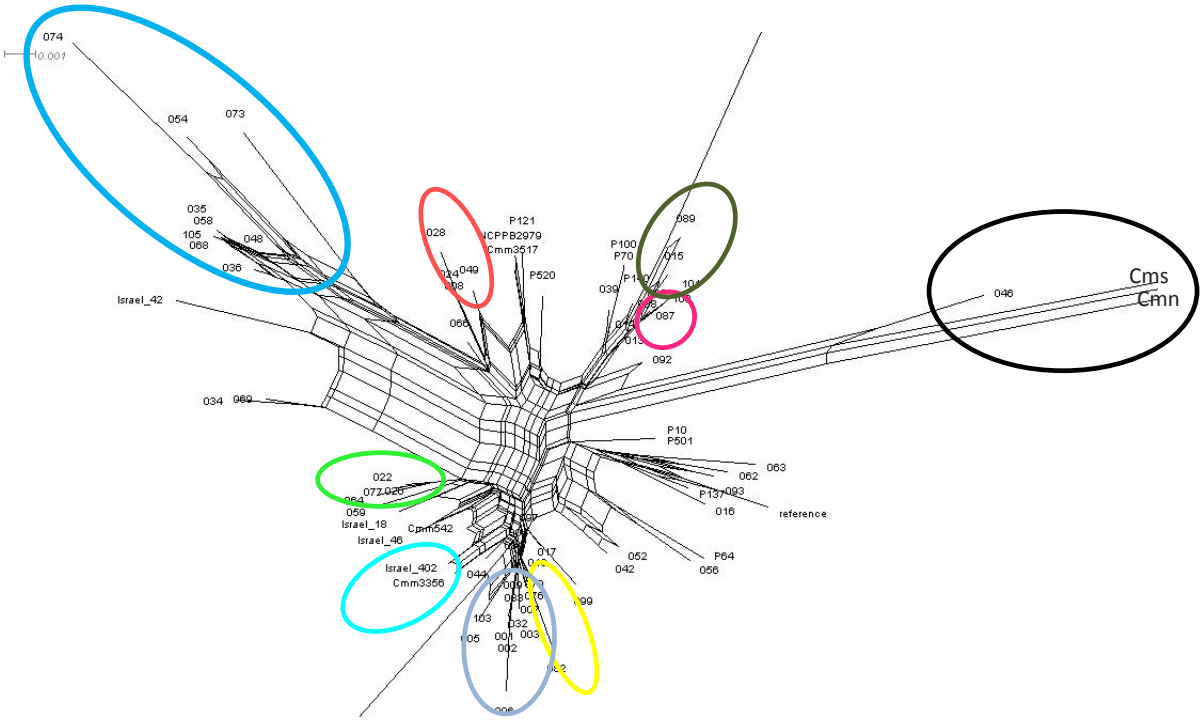


Figure 3: Split network analysis of concatenated data of 8 genes. Colored circles representing eight clonal complexes indicated in ML tree. Black circle represents the non-tomato host group.

Grouping of strains by split network analysis gave similar results as the clonal complex analysis and the ML tree. The non-tomato host strains (*Cms*, *Cmn* and strain 46) grouped in the black colored circle (Fig. 3), which was closely located to strain 92. The strains from Serbia having a reduced level of virulence grouped in the ML tree and in the split network analysis and they were close to the non-tomato host group. The relation of the other strains was very similar to that as was indicated in the ML tree.

Discussion

We used eight genes for an MLST analysis of a collection of predominantly Turkish *Cmm* strains. *Chp*C, *PpaA* and *Tom*A, located on the PAI island of the *Cmm* genome, are considered to be disease-related genes, whereas *Lig*A, *Bip*A, *Sdh*A, *Kdp*A and *Gyr*B are housekeeping genes.

Although virulence factors of *Cmm* are found to be located on plasmids, called pat-1 and ceIA, their presence does not correlate well with virulence [11]. Moreover, plasmid exchange is very frequent between bacterial strains. Thus, previously identified plasmid originated virulence genes were not chosen for characterization of our collection. But genes that are located on PAI island, *Chp*C, *PpaA* and *Tom*A, were thought to be involved in virulence [87] and were absent in *Cmm* like non virulent strains [165]. Consequently, our gene selection based on their involvement in virulence is more appropriate than plasmid originated virulence genes. The genetic diversity within our 108 *Cmm* strains was relatively high for both individual genes as well as for concatenated data (0.0065) compared to other studies [12]. To test whether a significant diversity reduction occurred in our *Cmm* population, three independent tests (Table 1) were used. The tests indicated negative values for all but one individual gene (*Sdh*A) which is an indication for a low level of genetic diversity. The negative value is due to a selective sweep, purifying selection or population expansion [156]. Since the use of hybrid seeds started to increase in Turkey in the 1990s when Turkey moved towards professional agriculture, the occurrence of *Cmm* via contaminated seeds has spread quickly which might be the reason for population expansion. Another reason for a negative value can be a sampling bias, because all strains were obtained after an outbreak and therefore we have collected only virulent strains. A Tajima test is used to test neutrality of genes (selection forces) but its assumption doesn't hold always for neutrality. To determine the selection forces acting on the genes, average frequencies of synonymous substitutions per potential synonymous site (Ks) and non-synonymous substitutions per potential non-synonymous site (Ka) are measured. A general concept of population genetics is that housekeeping genes are under stabilizing selection and disease genes are under positive selection. The Ka/Ks ratio indicated that the housekeeping genes in this study had a value lower than one, which might be an indication of stabilizing selection forces acting on those genes or a population expansion event. Based on Ka/Ks estimates, *PpaA* was the only gene with a Ka/Ks value higher than one indicating a positive selection. In our study the disease-related genes *ChpC* and *TomA* do not comply with this concept. This might be explained by the fact that these disease related genes may be involved in pathogenicity but are not an absolute indication of pathogenicity [166]. It is important to choose proper genes with sufficient genetic variability to be able to use them for intra-species genetic analysis since in some case only 3 polymorphic sites were found in 7 housekeeping genes [167]. The genes that we have chosen had a higher diversity compared to other genes which were used to characterize *Cmm* [12].

eBURST analysis detected few ST clonal complexes and many singletons; similar results were found in other *Cmm* characterization studies [12]. Although the 63 unique ST show a high heterogeneity, most of the STs were connected to each other by means of DLVs where intermediate units (SLVs) were missing making it not possible to connect them to the same clonal complex. Based on eBURST group definition, most strains can be grouped into six units

and most STs seem to be connected by eBURST grouping although SLVs are missing. This model of strain relation fits with a population in which a selective sweep or rapid population expansion have been the main diversifying forces [168]. Most clonal complexes in this study were not related to a specific year or location and therefore not the result of the same tomato hybrid varieties growing in different fields and containing the same *Cmm* strains.

Phylogenetic signals of a gene tree can be quantified based on the number of visible clonal complexes at the edge of lineages. Phylogenetic signals were found for all genes but the signal varied. A weak phylogenetic signal can be the result of a recombination in these genes [169, 170] because a recombination can interfere with the phylogenetic signal between genes and thus an incongruent signal between different gene trees can occur [170]. The relation of nucleotide diversity and haplotype diversity is shown (Fig. 1). Assuming a mutational model, nucleotide diversity and allelic diversity should show a positive correlation. We have detected an uneven positive correlation between these parameters with also supportive information of both recombination and mutation events on *Cmm* bacterial evolution history as depicted in other bacterial species [171]. When we consider the diversifying of clones from founder strains within clonal complexes, we see a 1.4 ratio (14 recombination events and 10 mutation events). This ratio is very low compared to other bacteria [172, 173] in which this ratio was at least 15. Based on three parameters: partly congruence of gene tree, relation between nucleotide and haplotype diversity and recombination ratio, we can conclude that recombination and mutation have played an equal or almost equal role in *Cmm* evolution.

The maximum likelihood tree of concatenated loci separated the non-tomato host group from the rest. All clonal complexes were visible at the edge of lineages in ML tree. The phylogenetic relation of the Serbian groups was similar as in the previous study [13]. All these parameters are indicating that the ML tree based on MLST data were reliable. Bootstrap values supporting tree branching were low in the ML tree which can be due to low diversity or due to recombination size smaller than the lengths of sequences used for the construction of gene trees, then these may be poorly supported statistically because different parts of genes have different evolutionary histories [170].

Split network analysis has resulted in very similar results as the ML tree. All clonal complexes and their relation to other strains (Serbian, Israel collection) were similar (Fig. 4). Split network analysis is very similar to PCA analysis and this analysis is used for visualization of genetic relation of organisms on which recombination has a strong effect on gene evolution and tree construction by a bifurcating method is not appropriate [168]. In our study however, where recombination and mutation have almost equal levels of impact on *Cmm* evolution the split network analysis has resulted in similar results as the bifurcating tree construction which is additional proof of an equal effect of recombination and mutation on *Cmm* evolution.

MLST data can be used to reveal evolutionary relations on species or subspecies levels. This analysis is suitable for conclusions on long term epidemiology (global epidemiological relation) but might be unsuitable for short term epidemiology since genetic variation accumulates slowly and strains might be indistinguishable [169]. MLST analysis has been successful in revealing the relation of the species and subspecies level of *Cmm* [150]. Although MLST analysis is used for studies on evolutionary relations within and between species, we have some indications that the MLST technique can also be used to get an idea about the virulence level of *Cmm* strains. The best subset of genes which are suitable for characterization of *Cmm* by virulence level or host is not known yet. Disease related genes are usually not included in MLST analysis [169], we used genes which are related to epidemiology but they might not be directly related to disease development. Adaptation to a particular host requires, for microbial organisms, metabolomic changes in which changes occur through housekeeping genes. Therefore, MLST data can be used to distinguish strains which have adapted to different hosts. However, a MLST analysis can also be used to distinguish strains with different virulence levels. Our study showed that low virulent strains and strains that specialized on different host have a tendency to group. However, a more comprehensive analysis is needed, including more strains with different levels of virulence.

Despite the clear ST clonal complexes determined by the clonality analysis and grouping those the network and ML tree analyses identified only two major populations. The results show a high genetic diversity primarily between *Cmm* and the other *Cm* spp., but a low genetic diversity within *Cmm* strains. Although some clonal complexes were structured in different subpopulations, the genetic diversity among the different subgroups was not high enough to support a division in more subpopulations.

Analysis of bacterial MLST data should be handled in several ways depending on clonality and diversifying mechanisms of the organism under investigation [148]. In our study, we have elaborated our data with care using clonal complex analysis, split network analysis, maximum likelihood of sequence data and structural analysis. We have started our analysis with estimating the degree of clonality in our collection [168]. After determining clonal complexes and determining recombination and mutation effects, the bifurcating tree phylogenetic approach has been applied. Since we have detected incongruent signals in the individual gene trees, network analysis and structure analysis were performed to complete the phylogenetic picture. By combining all approaches, we were able to show the relation of our *Cmm* strains to each other and to global strains in the most appropriate way. Due to the low level diversity and the effect of recombination in our *Cmm* collection, the phylogenetic tree of concatenated data that was obtained with maximum likelihood analysis was poorly supported by bootstrap analysis [168] and structure analysis of population did not or was weakly supported by the result of the other analyses.

For better understanding relationship between clonal complexes and disease, a larger collection of *Cmm* strains including hyper virulent, reduced virulent and non-virulent ones should be examined. The success of MLST for discriminating this phenomena is depending on gene subset that are chosen.

Table 3: *Cmm* strains used in this study

Code Number	Date of Isolation	Host Plant	Location	Collector
1	1996	Tomato	Erdemli-Mersin	S. Tokgönül
2	1996	Tomato	Erdemli-Mersin	S. Tokgönül
3	1996	Tomato	Erdemli-Mersin	S. Tokgönül
4	1996	Tomato	Erdemli-Mersin	S. Tokgönül
5	1996	Tomato	Erdemli-Mersin	S. Tokgönül
6	1996	Tomato	Erdemli-Mersin	S. Tokgönül
7	1996	Tomato	Erdemli-Mersin	S. Tokgönül
8	1996	Tomato	Erdemli-Mersin	S. Tokgönül
9	1996	Tomato	Erdemli-Mersin	S. Tokgönül
10	1996	Tomato	Erdemli-Mersin	S. Tokgönül
11	1996	Tomato	Erdemli-Mersin	S. Tokgönül
12	1996	Tomato	Erdemli-Mersin	S. Tokgönül
13	1996	Tomato	Erdemli-Mersin	S. Tokgönül
14	1996	Tomato	Erdemli-Mersin	S. Tokgönül
15	1996	Tomato	Erdemli-Mersin	S. Tokgönül
16	1996	Tomato	Erdemli-Mersin	S. Tokgönül
17	1996	Tomato	Erdemli-Mersin	S. Tokgönül
18	1996	Tomato	Erdemli-Mersin	S. Tokgönül
19	1996	Tomato	Antalya	S. Tokgönül
20	1996	Tomato	Antalya	S. Tokgönül
21	1996	Tomato	Antalya	S. Tokgönül
22	1996	Tomato	Adana	S. Tokgönül
23	1996	Tomato	Adana	S. Tokgönül
24	1997	Tomato	Erdemli- Mersin	S. Tokgönül
25	1997	Tomato	Erdemli- Mersin	S. Tokgönül
26	1997	Tomato	Anamur- Mersin	S. Tokgönül
27	1998	Tomato	Adana	Y. Aysan
28	1998	Tomato	Adana	Y. Aysan
29	1998	Tomato	Adana	Y. Aysan
30	1998	Tomato	Adana	Y. Aysan
31	1998	Tomato	Adana	Y. Aysan
32	2002	Tomato	Tarsus/ Mersin	Y. Aysan
33	2002	Tomato	Antalya	Y. Aysan
34	2002	Tomato	Antalya	Y. Aysan

35	2004	Tomato	Artvin	Y. Aysan
36	2004	Tomato	Artvin	Y. Aysan
37	2004	Tomato	Dikili/İzmir	Y. Aysan
38	2004	Tomato	Dikili/İzmir	Y. Aysan
39	2004	Tomato	Adana	R.Yildiz
40	2004	Tomato	Adana	R.Yildiz
41	2004	Tomato	Adana	R.Yildiz
42	2004	Tomato	Tarsus/Mersin	R.Yildiz
43	2005	Tomato	ŞahmurduKöyü/ Mersin	R.Yildiz
44	2005	Tomato	ŞahmurduKöyü/ Mersin	R.Yildiz
45	2005	Tomato	TapureliKöyü/Mersin	R.Yildiz
46	2005	Eggplant	Aydıncık/ Mersin	R.Yildiz
47	2006	Tomato	Antalya	Y. Aysan
48	2006	Tomato	Mersin	Y. Aysan
49	2006	Tomato	Antalya	Y. Aysan
50	2007	Tomato	Adana	Y. Aysan
51	2007	Tomato	Erdemli- Mersin	R.Yildiz
52	2007	Tomato	Adana	Y. Aysan
53	2007	Tomato	Adana	Y. Aysan
54	2007	Tomato	Cicik/Mersin	R.Yildiz
55	2007	Tomato	Cicik/Mersin	R.Yildiz
56	2007	Tomato	Cicik/Mersin	R.Yildiz
57	2007	Tomato	Erdemli- Mersin	R.Yildiz
58	2007	Tomato	Erdemli- Mersin	R.Yildiz
59	2008	Tomato	TapureliKöyü/Mersin	R.Yildiz
60	2008	Tomato	Ödemiş, İzmir	Y. Aysan
61	2009	Tomato	Adana	Y. Aysan
62	2010	Tomato	Antalya	Y. Aysan
63	2010	Tomato	Antalya	Y. Aysan
64	2010	Tomato	Antalya	Y. Aysan
65	2010	Tomato	Antalya	Y. Aysan
66	2010	Tomato	Antalya	Y. Aysan
67	2010	Tomato	Antalya	Y. Aysan
68	2010	Tomato	Erdemli- Mersin	Y. Aysan
69	2010	Tomato	Erdemli- Mersin	Y. Aysan
70	2010	Tomato	Erdemli- Mersin	Y. Aysan

71	2010	Tomato	Erdemli- Mersin	Y. Aysan
72	2010	Tomato	Erdemli- Mersin	Y. Aysan
73	2010	Tomato	Erdemli- Mersin	Y. Aysan
74	2010	Tomato	Erdemli- Mersin	Y. Aysan
75	2010	Tomato	Erdemli- Mersin	Y. Aysan
76	2010	Tomato	Erdemli- Mersin	Y. Aysan
77	2010	Tomato	Erdemli- Mersin	Y. Aysan
78	2010	Tomato	Erdemli- Mersin	Y. Aysan
79	2010	Tomato	Erdemli- Mersin	Y. Aysan
80	2010	Tomato	Erdemli- Mersin	Y. Aysan
81	2010	Tomato	Erdemli- Mersin	Y. Aysan
82	2010	Tomato	Erdemli- Mersin	Y. Aysan
83	2010	Tomato	Tokat	Y. Yanar
84	2010	Tomato	Tokat	Y. Yanar
85	2010	Tomato	Tokat	Y. Yanar
86	2010	Tomato	Tokat	Y. Yanar
87	2010	Tomato	Tokat	Y. Yanar
88	2010	Tomato	Tokat	Y. Yanar
89	2010	Tomato	Erdemli- Mersin	Y. Aysan
90	2010	Tomato	Erdemli- Mersin	Y. Aysan
91	2010	Tomato	Erdemli- Mersin	Y. Aysan
92	2010	Tomato	Tokat	Y. Yanar
93	2010	Tomato	Tokat	Y. Yanar
94	2010	Tomato	Tokat	Y. Yanar
95	2010	Tomato	Tokat	Y. Yanar
96	2010	Tomato	Tokat	Y. Yanar
97	2010	Tomato	Erdemli- Mersin	Y. Aysan
98	2010	Tomato	Erdemli- Mersin	Y. Aysan
99	2010	Tomato	Tokat	Y. Yanar
100	2010	Tomato	Erdemli- Mersin	Y. Aysan
101	2010	Tomato	Erdemli- Mersin	Y. Aysan
102	2010	Tomato	Erdemli- Mersin	Y. Aysan
103	2010	Tomato	Tokat	Y. Yanar
104	2012	Tomato	Adana	Y. Aysan
105	2012	Tomato	Adana	Y. Aysan
106	2012	Tomato	Adana	Y. Aysan
107	2012	Tomato	Adana	Y. Aysan
108	2012	Tomato	Adana	Y. Aysan
Cmm 542		Tomato	Wageningen	

*Cmm*3356		Tomato	Wageningen	
*Cmm*3517		Tomato	Wageningen	
NCPP2979	1957	Tomato	Hungary	
P10	2006	Tomato	Serbia	
P64	2006	Tomato	Serbia	
P70	2006	Tomato	Serbia	
P137	2007	Tomato	Serbia	
P140	2007	Tomato	Serbia	
P521	2008	Tomato	Serbia	
P123	2007	Tomato	Serbia	
Israel_18	1997	Tomato	Israel	
Israel_42	2001	Tomato	Israel	
Israel_46	2001	Tomato	Israel	
Israel_402		Tomato	Israel	

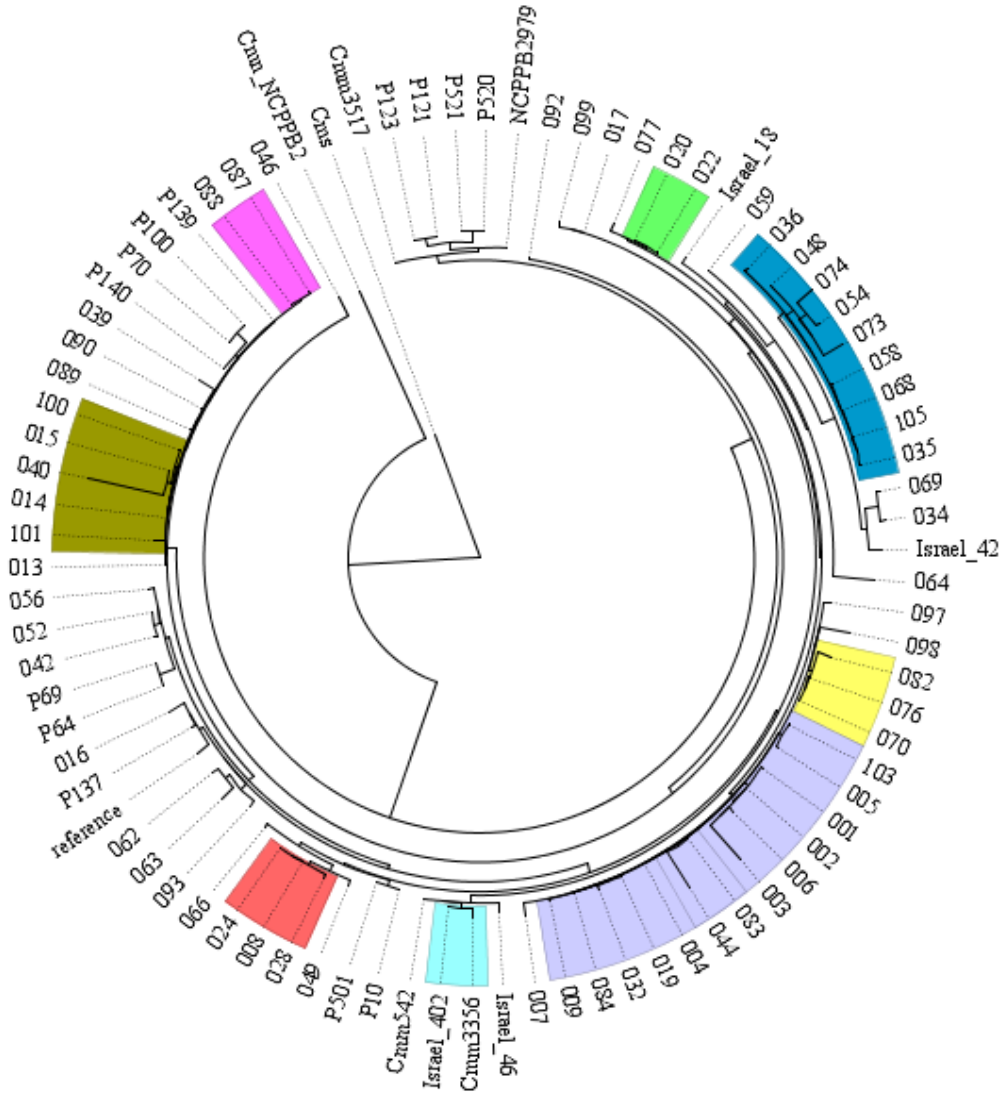


Figure 4: Maximum likelihood of concatenated data of eight genes. Colored groups are indicating clonal complexes.

Chapter 7

General Discussion

General Discussion

Clavibacter michiganensis subsp. *michiganensis* (*Cmm*) is an aerobic non-sporulating gram-positive plant pathogenic bacterium and the causal agent of bacterial canker in tomato. It is considered the most harmful bacterial threat for tomato. The disease was for the first time described about 100 years ago in Michigan, USA [1]. Attempts for breeding resistant varieties started in the 1960s [60] and although cultivars with partial resistance were introduced those cultivars were commercially not successful. This partial resistance originated from *S. lycopersicum* line Bulgaria-12 and the inheritance of this resistance was polygenic (Chapter 1). Other reported resistances were also polygenically controlled [6, 54, 69, 73, 74]. *S. arcanum* LA2157 is the best resistance source, it performs well under multi environmental conditions with different strains (Chapter 2 and 4). This Solanum species is genetically quite distantly related to *S. lycopersicum* and therefore difficult to cross and there is additional technology, such as embryo rescue, needed to make these crosses successful (Chapter 4). As a conclusion one can state that although several reported *Cmm* resistance sources are available, still there are no cultivars on the market containing a sufficient level of *Cmm* resistance.

After infection it takes one to three months to observe disease symptoms, the actual time depends on environmental conditions, the genetic background of the tomato and the developmental stage of the infected plant. Symptoms become visible when the bacteria have already spread throughout the plant and at this stage it is too late to stop the disease by means of chemical treatments. Removing infected plants, using clean, disinfected tools for cultural practices and applying copper based chemicals are the only management measures that can reduce the spread of the bacteria from plant to plant [3, 22]. Bacteria can also end up in the soil or somewhere in a greenhouse where soilless farming is practiced. Without a very thorough cleaning of the soil and/or greenhouse these bacteria will be the cause of infections in the following years [174]. *Cmm* is seed transmissible [17, 8, 175] and infected seeds are the main vectors of *Cmm*. The infection level can play a role in the speed of disease progress, highly contaminated seed batches will cause a severe epidemic. But also a low level of contamination, even only a few bacteria in a few seeds, can be a primary source [17] which can result in a serious epidemic by means of secondary spread [176]. According to strict rules seed companies have to sell *Cmm* free seeds. Contaminated seed lots have to be destroyed, in case they were already sold the companies have to pay high fines. Because of the long phase without symptoms and the seed transmission *Cmm* is under international quarantine regulations with zero tolerance [108].

To reliably monitor *Cmm* infection, sensitive and fast diagnostic tools are needed. We have discussed different detection methods with their advantages and disadvantages (Chapter 1). Detection methods are generally divided into four groups: serological, DNA based, bioassays

and dilution plating. Considering the combination of speed and sensitivity, serological methods can become the gold standard but costs and reliability are still major problems. DNA based methods are relatively new and improving. In Chapter 2, we have reported an improved TaqMan PCR protocol which can be used to identify and quantify *Cmm*. We have used this TaqMan protocol to quantify *Cmm* concentrations in individuals of a population. But still there is detection limit for our TaqMan assay which is around 100 to 1000 bacteria in one ml of plant extract. We recommend checking this improved version of TaqMan in contaminated seed batches in order to monitor seed transmission. By using lysosomal enzymes, the detection level of bacteria by TaqMan is increased tenfold [177]. The implementation of lysosomal enzymes in our TaqMan *Cmm* detection protocol might increase the sensitivity of detection to 10 cfu/ml. Generally speaking, DNA based techniques can be quick and reliable, but the sensitivity of the methods is currently not high enough to detect very low levels of bacteria which is needed to be sure of complete absence. Dilution plating is time consuming but the use of semi-selective mediums for dilution plating is still considered the most reliable and sensitive technique. In practice, none of these detection methods are good enough to be used as the only one and combinations of different methods are advised. Hopefully there will be in the near future improvements of sensitivity of the DNA based methods and of the reliability of serological methods.

As we mentioned above, the disease is only recognized when symptoms become visible and at that moment no cure is possible anymore. A way to control the disease and reduce the risks for the growers is to use tomato cultivars with *Cmm* resistance. So far, there are no *S. lycopersicum* cultivars with an acceptable level of resistance, and resistance has only been found in related wild species of tomato (Chapter 1). The screening for resistance until now was mainly based on wilting severity. Bacterial concentration and stem discoloration were not considered, only a few exceptions have been reported in which bacterial concentrations in the plant were investigated [58, 59]. We have used our improved TaqMan PCR to measure bacterial concentrations. In doing this we saw that in the more resistant genotypes significantly less bacteria were present than in the susceptible ones. However, no correlation was found between the different levels of resistance and bacterial concentration. Similar results have been reported by other researchers [58, 59] suggesting that bacterial inhibition is one of the resistance mechanisms but not the only one. The presence and concentration of bacteria in seeds is also an important parameter for resistance. The transmission rate to the seeds is an important trait which can be of importance for seed companies as well and it should be elaborated intensively. In our research, we have collected seeds of resistant accessions as well as seeds from a recombinant inbred line population. With the improved version of the BioTaqMan PCR technique it is now feasible to measure the transmission level to seeds. This also opens the door for genetic studies concerning seed transmission of *Cmm*.

In Chapters 3 and 4, we describe two mapping populations. One is based on a recombinant inbred line population originating from a cross between *S. pimpinellifolium* and *S. lycopersicum* followed by repeated selfings and another was an F2 population originating from a cross between *S. arcanum* and *S. lycopersicum.* Due to the high level of available Single Nucleotide Polymorphisms and the availability of a high quality tomato sequence, marker availability was no issue in the genetic studies. A genetic analysis is required in order to understand how many resistance factors differ between the parents of the crosses. In Chapter 1, we listed a number of genetic analyses and the interactions of QTLs which can be complementary or additive. In Chapters 3 and 4, we have described and identified additive effects of QTLs. Almost all genetic analyses of *Cmm* resistance done so far indicate a polygenic inheritance [6, 54, 68, 72-74, 117, 178-180]. A previously reported genetic analysis based on the resistance in *S. arcanum* LA2157 resulted in three QTL regions [73] and a genetic analysis based on the resistance of *S. pimpinellifolium* G1.1554 showed that at least 2 QTL were involved in getting lower levels of wilting. Most studies use only the level of wilting as a descriptor for the level of resistance [6, 57, 58, 67]. In our studies we have dissected the effects of *Cmm* on tomato in three components; wilting, bacterial titer and stem discoloration. Based on these three parameters five important QTLs on five different chromosomes were identified. In future experiments we would like to add more parameters like seed transmission level, morphology of the resistant plants and physiological parameters (see below).Traditional QTL approaches, using only the main (visible) phenotype fail to capture the dynamic nature of the disease resistance. Dissecting the effects of *Cmm* infection allows the further unravelling of all factors playing a role in higher or lower levels of resistance. To understand complex traits such an approach has been successfully used in plants [124], animals and humans [181]. This allows us to identify the number of genes involved in the process, interaction of those genes(epistasis), chromosomal location and genetic effects of those genes, and the expression of alleles in specific environments. Using a combination of a genetical genomic approach with QTL mapping of different resistance parameters might make it possible to find regulatory regions of genes involved in different parameters and to detect networks between the different biological processes. In our study (Chapter 3), we have detected environment specific QTL for wilting, stem discoloration and bacterial titer. Here dissecting enabled us to capture the dynamics of different process under different conditions. A genetic analysis of such resistance parameters by a multitrait mixed model approach is a more powerful way rather than elaborating resistance parameters separately by means of single QTL analysis (Chapter 3). Dissecting a complex trait such as *Cmm* resistance in tomato, will enable us to better understand the resistance mechanism behind the trait.

In Chapter 4, we have saturated the QTL regions in an F_2 of the cross *S. lycopersicum* x *S. arcanum* LA2157without increasing the population size. DNA isolation of this population was done 20 years ago and the DNA was stored at -20 ˚C. We were successful in new marker

development with KASPar and High Resolution Melting (HRM) techniques. Both technologies enabled us to place more molecular markers on the 1999 genetic map which was based on RFLP markers. The QTL intervals of all three QTLs could be more precisely determined, which was especially the case for the major QTL on Chromosome 7. For further fine mapping it will be necessary to use other populations or to increase the population size. The availability of the sequence of *S. arcanum* LA2157 makes it possible to find numerous markers in all the regions under investigation. For further studies we started the development of Nearly Isogenic Lines (NILs), marker assisted background selection did speed up this process by one year (two generations). Since *S. arcanum* and *S. lycopersicum* are distantly related species and difficult to cross we had to use embryo rescue. Embryo rescue was also needed in the first backcrosses of the NIL development. The Nearly Isogenic Lines (NILs) can be the starting point for recombinant screening and fine mapping but can also be used for obtaining combi-NILs in which NILs that harbor different QTL are crossed and NILs containing more than one QTL are obtained. The availability of NILs and combi-NILs will make it possible to study the mechanisms behind *Cmm* resistance more extensively. The availability of thousands of genetically identical seeds makes it possible to study differences in the plant pathogen interactions if different strains of the pathogen are used. NILs and combi-NILs can also be used to study plant pathogen interactions in different environments and conditions [77]. Validation and fine mapping of QTLs responsible for disease resistance and important agronomic traits using NILs and sub-NILs has been successfully applied in plants [182, 183]. One of the pitfalls in making NILs for validation of QTLs are inbreeding depression and self-incompatibility [184] consequently some QTLs might be lethal in one of the homozygous states. But by using NILs and sub-NILs, pleiotropy might get distinguishable from close linkage [183]. In our study the QTLs responsible for differences in bacterial titer and stem discoloration overlapped with the QTLs for wilting suggesting pleiotropic effects or linkage of genes. Fine mapping might show whether more genes are involved. Fine mapping is also necessary in order to reduce linkage drag. Besides fine mapping strategy in NILs, high resolution GWAS mapping has also been used for fine mapping purpose to discriminate pleiotropy from close linkage [185].

Combi-NILs are extremely useful to study interactions between QTLs. NILs can be used to confirm QTLs but before they are available we tried to confirm the QTLs in a Recombinant Inbred Line population based on the same parents (*S. lycopersicum* cv Solentos x *S. arcanum* LA2157). We couldn't confirm any of the three QTLs in this RIL population of 40 lines, instead a new QTL was found. Differences between the two experiments were the population size, the *Cmm* strains, the method and location of the screening. The F_2 population was screened with one single aggressive strain in a greenhouse in Wageningen whereas the second experiment (RILs) was carried out in a greenhouse in Antalya (southern part of Turkey) with a mix of fourteen different strains. In both experiments only the level of wilting was scored. Making RILs starting with the cross *S. lycopersicum* cv Solentos x *S. arcanum* LA2157 is difficult and starting

with 325 F_2 plants and single seed descent resulted in only 6 F_7 lines. This phenomena is due to inbreeding depression which is the result of homozygous state of deleterious alleles in a background different from the background of the wild relative. Large effects or combination of deleterious alleles might hamper germination (purging) or seed set [186] purging can be severe in adverse environmental conditions where condition is not suitable for plant natural growth [187]. Purging is more explicit with traits that effect early development such as germination success and seedling to adult viability [187]. Because major genes are expected to be expressed in early development inbreeding depression effect is likely to manifest itself early in embryos.

There is discussion about which strain(s) or the number of strains should be used for screening. *Cmm* strains exist in nature with different level of virulence [11, 150, 188]. Although fingerprinting of *Cmm* based on repetitive elements (Rep-PCR) have indicated four to six groups [11, 189], there was no strong correlation between this grouping and the virulence level of *Cmm* strains therefore classification of *Cmm* is generally made based on their virulence level. This revealed three distinct groups [189]: highly virulent, virulent and avirulent. Researchers usually prefer to use one strain (preferably the most aggressive one) in order to obtain the highest level of resistance in plant material [6, 15, 54, 73, 180, 190]. There is no information about *Cmm* strain-plant genotype interactions (even not with the strains in the highly virulent group). We have characterized our *Cmm* collection using sequence data of housekeeping genes (Chapter 5). We included three disease related genes in order to understand the evolutionary history of *Cmm*. Using different statistical approaches, we were able to reveal an evolutionary relation within our *Cmm* collection. By using this evolutionary relation network, we have connected the virulence level of our collection with our studied genes. Consequently, these genes can be used to predict the virulence levels of new strains. For resistance screenings strains representing populations with different virulence levels can be chosen.

In order to reveal *Cmm* resistance mechanism(s) in tomato, more attention should be given to phenotyping through evaluation of more components, and each component should be handled separately. Phenotyping traits should be extended to physiological components and more morphological traits should be included. Bacterial titer, stem discoloration, wilting and seed transmission level should be measured as separate components in order to shed light on the relation of these components. Bacterial inhibition is not the only resistance mechanism (Chapter2 and 3), but resistance mechanisms based on plant morphology and physiology should be considered as well [74-76]. Plant hormones like α-tomatin and ethylene [92, 98, 99] are thought to be involved in resistance to *Cmm*. Ethylene is known to play a role in softening plant tissue including stem tissue. In general ethylene treatment promote leaf senescence and fruit ripening [191]. Pathogen derived ethylene is involved in increasing susceptibility to herbivory in Arabidopsis [192] and tomato[193]. Ethylene is proven to be essential for the compatible reaction of gram negative bacteria *Pseudomonas syringae* and its host tomato via

type three secretion system (TTSS) [194]. The ethylene treatment effect depends often on timing, before pathogen attack ethylene can increase plant resistance however when applied/formed after pathogen attack it increases susceptibility [191]. The role of pathogen derived ethylene in susceptibility to *Cmm* in tomato through softening xylem tissue should be more extensively investigated. In our previous studies, we have observed reduced bacterial concentrations in different wild tomato sources [15]. Bacterial inhibition can be the result of antibacterial compounds such as the secondary plant metabolite α-tomatin that is known to be involved in basal defense of the plant and its concentration increases after pathogen attack [99]. Since *Cmm* has a tomatinase gene (tomA) whose protein breaks down α-tomatin, the interaction between α-tomatin and tomA should be considered in studying different levels of *Cmm* resistance. Bacteria can communicate via a mechanism called quorum sensing [195] by which they are able to change behavior, express virulence factors and form a biofilm structure [196, 197]. Quorum sensing causing behavior change and resulting in pathogenicity has been observed in gram positive human bacteria [198]. We think that *Cmm* is using quorum sensing to change their behavior from biotroph to necrotroph when the bacterial concentration reaches a certain level, probably 10^8cfu/g. If this level is reached bacteria start to attack the xylem vessel in tomato. Additionally, quorum sensing molecules of *Cmm* should be investigated and plant molecule(s) that may interfere with the quorum sensing system of bacteria should be studied as a physiological trait during phenotyping. Existence and quantity of those molecules should be investigated in wild tomato species. Morphological differences of the stem have been hypothesized to play a role in resistance [74] therefore a more detailed analysis of morphological differences in our sources is of utmost importance. Microscopic observations of stem morphology can add knowledge how the resistance mechanisms work. Knowledge about how to stop the transmission of *Cmm* into seeds would be a big step forward for breeders. If a resistance mechanism is based on differences in morphological structure then the risk is that the resistance will influence the growth of the plants which might be unacceptable for tomato growers. Plant phenotypes, for instance stem morphology similar to wild parents might affect plant yield especially in undetermined tomato by limiting of growth. In addition, in our *Cmm* resistance source we might face linkage drag causing unwanted fruit shape and color.

A genetical genomics approach [199] which takes advantage of combining genetics and genomics (genome wide gene expression, proteomics and metabolomics) in segregating populations can dissect the genetic mechanism behind *Cmm* resistance and find key genes that play a role in this mechanism. We have studied a RIL population of 100 individual lines derived from the cross between *S. lycopersicum* cv Moneymaker and *S. pimpinellifolium* GI. 1554. Of these 100 lines 60 have been completely resequenced. The genetic map based on this population is dense and a high similarity between this map and the published Kazusa map was found [126]. eQTL analyses in a RIL population of 100 is powerful due to 50 times replication of allelic state of each gene [199]. Thus by using this population, the power of detecting contrasts

will increase. In addition, genes responsible for ethylene pathway and α-tomatin biosynthesis, are good candidate genes to study. We also developed NILs carrying resistance QTLs from *S. arcanum* in *S. lycopersicum* cv Moneymaker background. Genetical genomic approach has successfully distinguished differentially expressed genes between NILs and its recurrent parent [200]. Expression studies in NILs have been used to find genes involved in late blight-tomato interactions [201]. Another example is a study on the interaction between stem root and soya where putative defense related genes in the phytohormone signalling pathways have been identified [202]. Genes involved in ethylene biosynthesis [193] and ethylene responsive transcription factors in NILs were successfully revealed in tomato - insect interactions [203]. Expression analysis has also been used to find candidate genes underlying quantitative disease resistance to leaf rust in barley [204]. Gene expression studies of NILs and combi-NILs also allow discriminating trans or cis acting genes more clearly. Besides gene expression, a metabolomic approach can be successfully used to determine genes underlying quantitative traits [205]. Untargeted metabolomic approach might reveal components or combinations of components that are related to phenotype [206].Since we have a well-studied recombinant inbred line population consisting of 100 lines and NILs from *S. arcanum*, these two different tools can be used to determine candidate genes and possible resistance mechanism(s) with –omics technology.

An alternative strategy to obtain *Cmm* resistance in tomato can be a genetic modification (GMO) approach. Serine protease is proven to be an essential protein involved in *Cmm* pathogenicity [85]. Serine protease inhibitors are considered to be effective for protection against pathogens [207, 208]. An attempt to control *Cmm in planta* by using a serine protease inhibitor protein has resulted in a somewhat reduced *Cmm* concentration [209], but this was not sufficient to control *Cmm*. Alternatively, quorum sensing interfering proteins can be used to prevent *Cmm* becoming virulent. This strategy has been widely used with different bacteria [210]. *Cmm* is able to produce HR on tobacco and *Mirabilis jalapa*, genes that are recognizing *Cmm* in these plant species can be identified and transferred to tomato to induce HR in tomato once *Cmm* enters tomato. Breeding companies are trying to use a GMO strategy to obtain *Cmm* resistance in tomato (personal communication with companies). But of course, GMO cultivars are still under discussion and only accepted in some countries [211].

As a conclusion and future prospect, due to the fast improvement of technology, we are closer to solve the Clavibacter problem in tomato than ever. Still, absolute resistance and complete absence of bacteria in resistant plants has not been found and probably no wild tomatoes harbor this kind of resistance. Effort should be given to phenotyping as parameters have been indicated above. Using these parameters, NILs or combi NIL should be evaluated and comparisons should be made with parents. By this way, QTL responsible for each component can be validated. Finally, NILs or combi-NILs can be evaluated under different conditions with

different aggressive strains using wilting as a final parameter. If such a resistance is still expressed in these advanced lines then lines which are similar to recurrent parent in terms of phenotypical characteristics and its performance can be advised to be used in breeding program. Genetical genomic approach can be taken in these sub-NIL population of these lines. In addition, genetical genomic approach can be taken in independent population, RILs derived from *S. pimpinellifolium*, and lines which had good performance can be used for backcrossing for NIL development. If such a progress is accomplished then combi-NIL approach can be taken using QTL from different species, *S. pimpinellifolium* and *S. arcanum*. With this study, we have made progress towards understanding the *Cmm* problem and made big steps in the development of advanced breeding material. To understand *Cmm* and its interaction with the host will make a contribution to understand a complex problem. We hope that in the future genes/alleles and the mechanism(s) behind the described resistance will be known and that our study made a contribution to this.

References

1. Smith EF: **A new tomato disease of economic importance**. Science 1910, 31:794-796.

2. Gartemann KH, Abt B, Bekel T, Burger A, Engemann J, Flugel M, Gaigalat L, Goesmann A, Grafen I, Kalinowski J *et al*: **The genome sequence of the tomato-pathogenic actinomycete Clavibacter michiganensis subsp. michiganensis NCPPB382 reveals a large island involved in pathogenicity**. *J Bacteriol* 2008, **190**(6):2138-2149.

3. de Leon L, Siverio F, Lopez MM, Rodriguez A: **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 Protection* 2008, **27**(9):1277-1283.

4. Carlton WM, Braun EJ, Gleason ML: **Ingress of Clavibacter michiganensis subsp michiganensis into tomato leaves through hydathodes**. *Phytopathology* 1998, **88**(6):525-529.

5. Strider DL: **Bacterial canker of tomato caused by Corynebacterium michiganense: a literature review and bibliography**. *Technical Bulletin North Carolina Agricultural Experiment Station* 1969(No. 193).

6. Poysa V: **Evaluation of Tomato Breeding Lines Resistant to Bacterial Canker**. *Canadian Journal of Plant Pathology-Revue Canadienne De Phytopathologie* 1993, **15**(4):301-304.

7. Cass Smith WP, Goss M: **Bacterial canker of tomatoes**. *Journal Department of Agriculture Western Australia* 1946, **23**:147-156.

8. Eichenlaub R, Gartemann KH, Burger A: **Clavibacter michiganensis , a group of gram-positive phytopathogenic bacteria**. In: *Plant-Associated Bacteria.* Springer Netherlands; 2006: 385-421.

9. Alvarez AM, Kaneshiro WS, Vine BG: **Diversity of *Clavibacter michiganensis subsp.michiganensis* population in tomato seeds: What is significance**. *Acta Hort (ISHS)* 2004, **695**:205-214.

10. Kawaguchi A, Tanina K, Inoue K: **Molecular typing and spread of Clavibacter michiganensis subsp michiganensis in greenhouses in Japan**. *Plant Pathology* 2010, **59**(1):76-83.

11. Kleitman F, Barash I, Burger A, Iraki N, Falah Y, Sessa G, Weinthal D, Chalupowicz L, Gartemann KH, Eichenlaub R *et al*: **Characterization of a Clavibacter michiganensis subsp michiganensis population in Israel**. *European Journal of Plant Pathology* 2008, **121**(4):463-475.

12. Quesada-Ocampo LM, Landers NA, Lebeis AC, Fulbright DW, Hausbeck MK: **Genetic Structure of Clavibacter michiganensis subsp. michiganensis Populations in Michigan Commercial Tomato Fields**. *Plant Disease* 2012, **96**(6):788-796.

13. Milijašević-Marčić S, Gartemann K-H, Frohwitter J, Eichenlaub R, Todorović B, Rekanović E, Potočnik I: **Characterization of Clavibacter michiganensis subsp michiganensis strains from recent outbreaks of bacterial wilt and canker in Serbia**. *European Journal of Plant Pathology* 2012:1-15.

14. Mansfield J, Genin S, Magori S, Citovsky V, Sriariyanum M, Ronald P, Dow M, Verdier V, Beer SV, Machado MA *et al*: **Top 10 plant pathogenic bacteria in molecular plant pathology**. *Molecular plant pathology* 2012, **13**(6):614-629.

15. Sen Y, Zhu F, Vandenbroucke H, van der Wolf J, Visser R, van Heusden A: **Screening for new sources of resistance to *Clavibacter michiganensis subsp. michiganensis*(*Cmm*) in tomato**. *Euphytica* 2012:1-9.

16. Lelis FV, Czajkowski R, Souza R, Ribeiro D, Wolf J: **Studies on the colonization of axenically grown tomato plants by a GFP-tagged strain of Clavibacter michiganensis subsp. michiganensis**. *European Journal of Plant Pathology* 2014, **139**(1):53-66.

17. Chang RJ, Ries SM, Pataky JK: **Dissemination of Clavibacter-Michiganensis Subsp Michiganensis by Practices Used to Produce Tomato Transplants**. *Phytopathology* 1991, **81**(10):1276-1281.

18. Wolf vd JM, Zouwen vd PS, Ludeking DJW, Hamelink R, Schenk MF: **Onderzoeksverslag 'Distributie van Clavibacter michiganensis subsp. michiganensis in tomatenplanten'**. *Plant Research International* 2012, **Report 448**.

19. Ricker MD, Riedel RM: **Effect of Secondary Spread of Clavibacter-Michiganensis Subsp Michiganensis on Yield of Northern Processing Tomatoes**. *Plant Disease* 1993, **77**(4):364-366.

20. Kontaxis DG: **Leaf trichomes as avenues for infection by Corynebacterium michiganense**. *Phytopathology* 1962:1306-1307.

21. Fatmi M, Schaad NW: **Survival of Clavibacter michiganensis ssp michiganensis in infected tomato stems under natural field conditions in California, Ohio and Morocco**. *Plant Pathology* 2002, **51**(2):149-154.

22. Hausbeck MK, Bell J, Medina-Mora C, Podolsky R, Fulbright DW: **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* 2000, **90**(1):38-44.

23. Yang XE, Long XX, Ni WZ, Ye ZQ, He ZL, Stoffella PJ, Calvert DV: **Assessing copper thresholds for phytotoxicity and potential dietary toxicity in selected vegetable crops**. *Journal of environmental science and health Part B, Pesticides, food contaminants, and agricultural wastes* 2002, **37**(6):625-635.

24. Filippone MP, Diaz-Ricci JC, Castagnaro AP, Farias RN: **Effect of fragarin on the cytoplasmic membrane of the phytopathogen Clavibacter michiganensis**. *Mol Plant Microbe Interact* 2001, **14**(7):925-928.

25. Wittmann J, Eichenlaub R, Dreiseikelmann B: **The endolysins of bacteriophages CMP1 and CN77 are specific for the lysis of Clavibacter michiganensis strains**. *Microbiology* 2010, **156**(8):2366-2373.

26. Daferera DJ, Ziogas BN, Polissiou MG: **The effectiveness of plant essential oils on the growth of Botrytis cinerea, Fusarium sp. and Clavibacter michiganensis subsp. michiganensis**. *Crop Protection* 2002, **22**:39-44.

27. Baysal O, Gursoy Y, Ornek H, Duru A: **Induction of oxidants in tomato leaves treated with DL-beta-amino butyric acid (BABA) and infected with Clavibacter michiganensis ssp michiganensis**. *European Journal of Plant Pathology* 2005, **112**(4):361-369.

28. Baysal O, Soylu EM, Soylu S: **Induction of defence-related enzymes and resistance by the plant activator acibenzolar-S-methyl in tomato seedlings against bacterial canker caused by Clavibacter michiganensis ssp michiganensis**. *Plant Pathology* 2003, **52**(6):747-753.

29. Utkhede R, Koch C: **Biological treatments to control bacterial canker of greenhouse tomatoes**. *BioControl* 2004, **49**(3):305-313.

30. Müller J, Völksch B, Eisbein K, Griesbach E: **Induction of resistance to bacterial pathogens in the pathosystem tomato/Clavibacter michiganensis subsp. michiganensis III. Characterization of the resistance induction in tomato cell cultures**. *Zeitschrift für Pflanzenkrankheiten und Pflanzenschutz* 2000, **107** (6):561-573

31. Huang R, Tu JC: **Effects of nutrient solution pH on the survival and transmission of Clavibacter michiganensis ssp michiganensis in hydroponically grown tomatoes**. *Plant Pathology* 2001, **50**(4):503-508.

32. ISF: **Method for detection of *Clavibacter michiganensis subsp. michiganensis* on tomato seed**. *Internatuional Seed Federation Report* 2011.

33. ISHI: **Protocol for the Detection of Clavibacter michiganensis subsp. michiganensis on Tomato Seeds by Isolation on Media Version 3.** 2008

34. Franken AAJM, Kamminga GC, Snijders W, Van Der Zouwen PS, Birnbaum YE: **Detection of Clavibacter michiganensis ssp. michiganensis in tomato seeds by immunofluorescence microscopy and dilution plating**. *European Journal of Plant Pathology* 1993, **99**(3):125-137.

35. Kaneshiro W, Mizumoto C, Alvarez A: **Differentiation of Clavibacter michiganensis subsp. michiganensis from seed-borne saprophytes using ELISA, Biolog and 16S rDNA sequencing**. 2006, **116**(1):45-56.

36. Franken A, Kamminga G, Snijders W, Van der Zouwen P, Birnbaum Y: **Detection of Clavibacter michiganensis ssp. michiganensis in tomato seeds by immunofluorescence microscopy and dilution plating**. *European Journal of Plant Pathology* 1993, **99**(3):125-137.

37. Dreier J, Bermpohl A, Eichenlaup R: **Southern hybridization and PCR for specific detection of phytopathogenic Clavibacter michiganensis subsp. michiganensis**. *Phytopathology* 1995, **85**:462-468.

38. Schaad NW, Jones JB, Chun W: **Laboratory Guide For Identifiction Of Plant Pathogenic Bacteria**. St. Paul, Minn.: American Phytopathological Society; 2001.

39. Olivier V, Baloche A, Drouin A, Audusseau C, Paillard S, Soubelet H: **Internal methods comparison study and inter-laboratory study on Clavibacter michiganensis subsp. michiganensis in tomato seeds1**. *EPPO Bulletin* 2010, **40**(2):248-256.

40. Veena MS, van Vuurde JWL: **Indirect immunofluorescence colony staining method for detecting bacterial pathogens of tomato**. *Journal of Microbiological Methods* 2002, **49**(1):11-17.

41. Nemeth J, Vuurde JWLv: **Development of immunofluorescence colony staining (IFC) for detection of Xanthomonas campestris pv. vesicatoria and Clavibacter michiganensis subsp michiganensis in tomato seeds**. *Seed Science and Technology* 2006, **34**(1):85-100.

42. de Leon L, Siverio F, Rodriguez A: **Detection of Clavibacter michiganensis subsp michiganensis in tomato seeds using immunomagnetic separation**. *Journal of Microbiological Methods* 2006, **67**(1):141-149.

43. de Leon L, Rodriguez A, Lopez MM, Siverio F: **Evaluation of the efficacy of immunomagnetic separation for the detection of Clavibacter michiganensis subsp. michiganensis in tomato seeds**. *J Appl Microbiol* 2008, **104**(3):776-786.

44. Hadas R, Kritzman G, Klietman F, Gefen T, Manulis S: **Comparison of extraction procedures and determination of the detection threshold for Clavibacter michiganensis ssp michiganensis in tomato seeds**. *Plant Pathology* 2005, **54**(5):643-649.

45. Schaad NW, Frederick RD: **Real-time PCR and its application for rapid plant disease diagnostics**. *Canadian journal of plant pathology Revue Canadienne de phytopathologie* 2002, **24**(3):250-258.

46. Bach HJ, Jessen I, Schloter M, Munch JC: **A TaqMan-PCR protocol for quantification and differentiation of the phytopathogenic Clavibacter michiganensis subspecies**. *J Microbiol Methods* 2003, **52**(1):85-91.

47. Luo LX, Walters C, Bolkan H, Liu XL, Li JQ: **Quantification of viable cells of Clavibacter michiganensis subsp michiganensis using a DNA binding dye and a real-time PCR assay**. *Plant Pathology* 2008, **57**(2):332-337.

48. Fatmi M, Schaad NW: **Semiselective agar medium for isolation of Clavibacter michiganense subsp. michiganense from tomato seed**. *Phytopathology* 1988, **78**(1):121-126.

49. Bolkan HA, Waters CM, Fatmi M: **ISTA handbook on seed health testing. Zurich, Switzerland**. *ISTA* 1996, **sheet no 67**.

50. Chun WCC: **Identification and detection of Corynebacterium michiganense in tomato seed using the indirect enzime-linked immunosorbent assay**. *Honolulu, HI: University of Hawaii, MSc Thesis* 1982.

51. Ftayeh RM, von Tiedemann A, Rudolph KWE: **A New Selective Medium for Isolation of Clavibacter michiganensis subsp. michiganensis from Tomato Plants and Seed**. *Phytopathology* 2011, **101**(11):1355-1364.

52. Kaneshiro WS, Mizumoto CY, Alvarez AM: **Differentiation of Clavibacter michiganensis subsp michiganensis from seed-borne saprophytes using ELISA, Biolog and 16S rDNA sequencing**. *European Journal of Plant Pathology* 2006, **116**(1):45-56.

53. Alvarez AM, Kaneshiro WS, Vine BG: **Diversity of Clavibacter michiganensis subsp. michiganensis populations in tomato seeds: What is significance?** *Acta Hort(ISHS)* 2005, **695**:205-214.

54. Thyr BD: **Inheritance of Resistance to Corynebacterium michiganense in Tomato**. *Phytopathology* 1976, **66**:1116-1119.

55. Hassan AA, Strider DL, Konsler TL: **Application of cotyledonory symptoms in screening for resistance of tomato to bacterial canker and host range studied**. *Phytopathology* 1968, **58**:233-239.

56. Sotirova V, Achkova ZI: **Resistance of tomatoes to Corynebacterium michiganense (Smith) Jensen**. *XII Eucarpia Congress* 1989.

57. Sotirova V, Bogatsevska N, Stamova L: **Sources of resistance to bacterial diseases in tomato wild species**. *Acta Hort (ISHS)* 1994, **376**:353-360.

58. Francis DM, Kabelka E, Bell J, Franchino B, Clair DS: **Resistance to bacterial canker in tomato (Lycopersicon hirsutum LA407) and its progeny derived from crosses to L-esculentum**. *Plant Disease* 2001, **85**(11):1171-1176.

59. Thyr BD: **Resistance to Corynebacterium michiganense measured in six Lycopersicon accessions**. *Phytopathology* 1971, **61**:972-974.

60. Elenkov E: **Die Selektion von Tomaten auf Resistenz gegen die Bakterienwelke**. *Int Z Landwirt* 1965:594-597.

61. Thyr BD: **Pedigree of CmVF 232**. *Report of tomato genetics cooperative* 1972, **22**(52).

62. Emmatty DA, John CA: **Evaluation of Resistance to Bacterial Canker of H2990, a New Tomato Variety**. *Plant Disease Reporter* 1973, **57**(7):584-586.

63. Forster RL, Echandi E: **Relation of age of plants, temperature, and inoculum concentration to bacterial canker development in resistant and susceptible Lycopersicon spp**. *Phytopathology* 1973, **63**:773-777.

64. Kuriyama T, Kuniyasu K: **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 Ornam Crops Res Stn Japan* 1974, **A 1**:93-107.

65. Laterrot H, Brand R, Daunay MC: **La resistance à Corynebacterium michiganense chez la tomate**. *Étude bibliographique Ann Amélior Plantes* 1978, **28**:579-591.

66. Gardner RG, Shoemaker PB, Echandi E: **Evaluation of tomato lines for resistance to bacterial canker in North Carolina** *Tomato Genet CoopRep* 1990, **40**:10-12.

67. Crinò P, Veroness, P., Stamigna, C., Chiaretti, D., Lai, A., Bitti, M.E. and Saccardo, F.: **Breeding for resistance to bacterial canker in Italian tomatoes for fresh market**. *Acta Hort (ISHS)* 1995, **412**:539-545.

68. Lindhout P, Purimahua C: **Resistance against Corynebacterium michiganense found in Lycopersicon peruvianum**. *10th meeting Eucarpia Tomato working group, Pontecagnano, Italy* 1987.

69. Thyr BD: **Virulence of Corynebacterium michiganense Isolates on Lycopersicon Accessions**. *Phytopathology* 1972, **62**:1982-1084.

70. Lindhout P, Purimahua C: **Investigations on tomato breeding for resistance to Corynebacterium michiganense** *In: Book of poster abstracts, Part II XIIth EUCARPIA Congress, Science for Plant Breeding, Göttingen, Germany,* 1989:16–18.

71. Vulkova ZV, Sotirova VG: **Study of the three-genome hybridLycopersicon esculentum Mill. —L. chilense Dun. —L. peruvianum var 'humifusum' Mill. and its use as a source for resistance**. *TAG Theoretical and Applied Genetics* 1993, **87**(3):337-342.

72. Sandbrink JM, Vanooijen JW, Purimahua CC, Vrielink M, Verkerk R, Zabel P, Lindhout P: **Localization of Genes for Bacterial Canker Resistance in Lycopersicon-Peruvianum Using Rflps**. *Theor Appl Genet* 1995, **90**(3-4):444-450.

73. van Heusden AW, Koornneef M, Voorrips RE, Bruggemann W, Pet G, Vrielink van Ginkel R, Chen X, Lindhout P: **Three QTLs from Lycopersicon peruvianum confer a high level of resistance to Clavibacter michiganensis ssp michiganensis**. *Theor Appl Genet* 1999, **99**(6):1068-1074.

74. Kabelka E, Franchino B, Francis DM: **Two loci from Lycopersicon hirsutum LA407 confer resistance to strains of Clavibacter michiganensis subsp michiganensis**. *Phytopathology* 2002, **92**(5):504-510.

75. Coaker G, Francis D: **Mapping, genetic effects, and epistatic interaction of two bacterial canker resistance QTLs from Lycopersicon hirsutum**. *Theor Appl Genet* 2004, **108**:1047-1055.

76. Coaker GL, Meulia T, Kabelka EA, Jones AK, Francis DM: **A QTL controlling stem morphology and vascular development in Lycopersicon esculentum X Lycopersicon hirsutum (Solanaceae) crosses is located on chromosome 2**. *American Journal of Botany* 2002, **89**(12):1859-1866.

77. Chang RJ, Ries SM, Pataky JK: **Effects of Temperature, Plant-Age, Inoculum Concentration, and Cultivar on the Incubation Period and Severity of Bacterial Canker of Tomato**. *Plant Disease* 1992, **76**(11):1150-1155.

78. De Jong J, Honma S: **Inheritance of Resistance to Corynebacterium michiganense in the Tomato**. *Journal of Heredity* 1976, **67**(2):79-84.

79. Pine TS, Grogan RG, Hewitt WB: **Pathological Anatomy of Bacterial Canker of Young Tomato Plants**. *Phytopathology* 1955, **45**(5):267-271.

80. Wallis FM: **Ultrastructural histopathology of tomato plants infected with *Corynebacterium michiganense***. *Physiological Plant pathology* 1977, **11**:333-342.

81. Xu XL, Miller SA, Baysal-Gurel F, Gartemann KH, Eichenlaub R, Rajashekara G: **Bioluminescence Imaging of Clavibacter michiganensis subsp michiganensis Infection of Tomato Seeds and Plants**. *Applied and Environmental Microbiology* 2010, **76**(12):3978-3988.

82. Gartemann KH, Abt B, Bekel T, Burger A, Engemann J, Flugel M, Gaigalat L, Goesmann A, Grafen I, Kalinowski J *et al*: **The genome sequence of the tomato-pathogenic actinomycete Clavibacter michiganensis subsp michiganensis NCPPB382 reveals a large island involved in pathogenicity**. *Journal of Bacteriology* 2008, **190**(6):2138-2149.

83. Jahr H, Dreier J, Meletzus D, Bahro R, Eichenlaub R: **The endo-beta-1,4-glucanase CelA of Clavibacter michiganensis subsp michiganensis is a pathogenicity determinant required for induction of bacterial wilt of tomato**. *Molecular Plant-Microbe Interactions* 2000, **13**(7):703-714.

84. Dreier J, Meletzus D, Eichenlaub R: **Characterization of the plasmid encoded virulence region pat-1 of phytopathogenic Clavibacter michiganensis subsp michiganensis**. *Molecular Plant-Microbe Interactions* 1997, **10**(2):195-206.

85. Burger A, Grafen I, Engemann J, Niermann E, Pieper M, Kirchner O, Gartemann KH, Eichenlaub R: **Identification of homologues to the pathogenicity factor Pat-1, a putative serine protease of Clavibacter michiganensis subsp. michiganensis**. *Microbiol Res* 2005, **160**(4):417-427.

86. Eichenlaub R, Gartemann KH: **The Clavibacter michiganensis subspecies: molecular investigation of gram-positive bacterial plant pathogens**. *Annual review of phytopathology* 2011, **49**:445-464.

87. Stork I, Gartemann KH, Burger A, Eichenlaub R: **A family of serine proteases of Clavibacter michiganensis subsp. michiganensis: chpC plays a role in colonization of the host plant tomato**. *Molecular plant pathology* 2008, **9**(5):599-608.

88. Kaup O, Grafen I, Zellermann EM, Eichenlaub R, Gartemann KH: **Identification of a tomatinase in the tomato-pathogenic actinomycete Clavibacter michiganensis subsp. michiganensis NCPPB382**. *Mol Plant Microbe Interact* 2005, **18**(10):1090-1098.

89. Flugel M, Becker A, Gartemann KH, Eichenlaub R: **Analysis of the interaction of Clavibacter michiganensis subsp michiganensis with its host plant tomato by genome-wide expression profiling**. *J Biotechnol* 2012, **160**(1-2):42-54.

90. Chalupowicz L, Cohen-Kandli M, Dror O, Eichenlaub R, Gartemann KH, Sessa G, Barash I, Manulis-Sasson S: **Sequential expression of bacterial virulence and plant defense genes during infection of tomato with Clavibacter michiganensis subsp. michiganensis**. *Phytopathology* 2010, **100**(3):252-261.

91. Chalupowicz L, Zellermann EM, Fluegel M, Dror O, Eichenlaub R, Gartemann KH, Savidor A, Sessa G, Raki N, Barash I *et al*: **Colonization and Movement of GFP-Labeled Clavibacter michiganensis subsp michiganensis During Tomato Infection**. *Phytopathology* 2012, **102**(1):23-31.

92. Savidor A, Teper D, Gartemann KH, Eichenlaub R, Chalupowicz L, Manulis-Sasson S, Barash I, Tews H, Mayer K, Giannone RJ *et al*: **The Clavibacter michiganensis subsp. michiganensis-tomato interactome reveals the perception of pathogen by the host and suggests mechanisms of infection**. *Journal of proteome research* 2012, **11**(2):736-750.

93. Dreier J, Meletzus D, Eichenlaub R: **Characterization of the plasmid encoded virulence region pat-1 of phytopathogenic Clavibacter michiganensis subsp. michiganensis**. *Mol Plant Microbe Interact* 1997, **10**(2):195-206.

94. Henke JM, Bassler BL: **Bacterial social engagements**. *Trends Cell Biol* 2004, **14**(11):648-656.

95. Vu B, Chen M, Crawford RJ, Ivanova EP: **Bacterial extracellular polysaccharides involved in biofilm formation**. *Molecules* 2009, **14**(7):2535-2554.

96. Vandenbulk RW, Zevenhuizen LPTM, Cordewener JHG, Dons JJM: **Characterization of the Extracellular Polysaccharide Produced by Clavibacter-Michiganensis Subsp Michiganensis**. *Phytopathology* 1991, **81**(6):619-623.

97. Bermpohl A, Drier, J.,Bahro, R.,Eichenlaub, R.: **Exopolysaccharides in the pathogenic interaction of Clavibacter michiganensis subsp. michiganensis with tomato plants** *Microbiological Research* 1996, **151**(4):391-399.

98. Roldan-Arjona T, Perez-Espinosa A, Ruiz-Rubio M: **Tomatinase from Fusarium oxysporum f. sp. lycopersici defines a new class of saponinases**. *Mol Plant Microbe Interact* 1999, **12**(10):852-861.

99. Arwiyanto T, Sakata K, Goto M, Tsuyumu S, Takikawa Y: **Induction of tomatine in tomato [Lycopersicon esculenta] plant by an avirulent strain of Pseudomonas solanacearum**. *Annals of the Phytopathological Society of Japan* 1994, **60**(3): 288-294.

100. Balaji V, Mayrose M, Sherf O, Jacob-Hirsch J, Eichenlaub R, Iraki N, Manulis-Sasson S, Rechavi G, Barash I, Sessa G: **Tomato transcriptional changes in response to Clavibacter michiganensis subsp. michiganensis reveal a role for ethylene in disease development**. *Plant Physiol* 2008, **146**(4):1797-1809.

101. Lara-Ávila J, Isordia-Jasso M, Castillo-Collazo R, Simpson J, Alpuche-Solís Á: **Gene Expression Analysis during Interaction of Tomato and Related Wild Species with *Clavibacter michiganensis subsp. michiganensis***. *Plant Molecular Biology Reporter* 2012, **30**(2):498-511.

102. van Loon LC, Geraats BPJ, Linthorst HJM: **Ethylene as a modulator of disease resistance in plants**. *Trends in Plant Science* 2006, **11**(4):184-191.

103. Balaji V, Sessa G, Smart CD: **Silencing of Host Basal Defense Response-Related Gene Expression Increases Susceptibility of Nicotiana benthamiana to Clavibacter michiganensis subsp. michiganensis**. *Phytopathology* 2011, **101**(3):349-357.

104. Balaji V, Smart CD: **Over-expression of snakin-2 and extensin-like protein genes restricts pathogen invasiveness and enhances tolerance to Clavibacter michiganensis subsp. michiganensis in transgenic tomato (Solanum lycopersicum)**. *Transgenic research* 2012, **21**(1):23-37.

105. Coaker GL, Willard B, Kinter M, Stockinger EJ, Francis DM: **Proteomic analysis of resistance mediated by Rcm 2.0 and Rcm 5.1, two loci controlling resistance to bacterial canker of tomato**. *Molecular Plant-Microbe Interactions* 2004, **17**(9):1019-1028.

106. Gartemann KH, Kirchner O, Engemann J, Grafen I, Eichenlaub R, Burger A: **Clavibacter michiganensis subsp. michiganensis: first steps in the understanding of virulence of a Gram-positive phytopathogenic bacterium**. *J Biotechnol* 2003, **106**(2-3):179-191.

107. Lindhout P, Purimahua C: **investigation on tomato breeding for resistance to Corynebacterium michiganense**. *XII Eucarpia Congress* 1989.

108. Anonymous: **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**. *Official Journal of the European Communities* 2000, **L 169**(1):33.

109. Zhao WJ, Chen HY, Zhu SF, Xia MX, Tan TW: **One-step detection of Clavibacter michiganensis subsp. michiganensis in symptomless tomato seeds using a TaqMan probe** *Journal of Plant Pathology* 2007, **89**(3):349-351.

110. Koenraadt H, van Vliet A, Neijndorff N, Woudt B: **Improvement of semi-selective media for the detection of Clavibacter michiganensis subsp. michiganensis in seeds of tomato. (Abstr.)** *Phytopathology 99:S66* 2009.

111. Chun WCC: **Identification and detection of Corynebacterium michiganense in tomato seed using the indirect enzyme-linked immunosorbent assay**. *MSc thesis, University of Hawaii, Honolulu* 1982.

112. Santos MS, Cruz L, Norskov P, Rasmussen OF: **A rapid and sensitive detection of Clavibacter michiganensis subsp. michiganensis in tomato seeds by polymerase chain reaction**. *Seed science and technology* 1997, **25**(3):581-584.

113. Berendsen SMH, Koenraadt H, Woudt B, Oosterhof J: **The development of a specific Real-Time TaqMan for the detection of Calvibacter michiganensis subsp. michiganensis**. *APS-IPPC Meeting,Honolulu, Hawaii* 2011.

114. Peralta I, Spooner D, Knapp S: **Taxonomy of wild tomatoes and their relatives (Solanum sect. Lycopersicoides, sect. Juglandifolia, sect. Lycopersicon; Solanaceae)**. *Syst Bot Monogr* 2008, **84**:1.

115. Anonymous: **ISHI Protocol for the Detection of Clavibacter michiganensis subsp. michiganensis on Tomato Seeds by Isolation on Media Version 3.** 2008.

116. Van Steekelenburg NAM: **Resistance to Corynebacterium michiganense in tomato genotypes**. *Euphytica* 1985, **34**:245-250.

117. Thyr BD: **Evaluation of Tomato Seedlings in Greenhouse for Resistance to Bacterial Canker (Corynebacterium Michiganense)**. *Phytopathology* 1967, **57**(10):1009.

118. Voorrips R, Verkerke W, Finkers R, Jongerius R, Kanne J: **Inheritance of taste components in tomato**. *Acta Physiol Plant* 2000, **22**(3):259-261.

119. Viquez-Zamora M, Vosman B, van de Geest H, Bovy A, Visser R, Finkers R, van Heusden A: **Tomato breeding in the genomics era: insights from a SNP array**. *BMC Genomics* 2013, **14**(1):354.

120. Stam P: **Construction of integrated genetic linkage maps by means of a new computer package: Join Map**. *The Plant Journal* 1993, **3**(5):739-744.

121. Van Ooijen JW: **Software for the mapping of quantitative trait loci in experimental populations of diploid species** *Kyazma BV, Wageningen, Netherlands* 2009.

122. International V: **GenStat for Windows 14th Edition** *VSN International, Hemel Hempstead, UK* 2011, **www.GenStat.co.uk**.

123. Chibon PY, Schoof H, Visser RG, Finkers R: **Marker2sequence, mine your QTL regions for candidate genes**. *Bioinformatics* 2012, **28**(14):1921-1922.

124. Firdaus S, Van Heusden A, Harpenas A, Supena EDJ, Visser RGF, Vosman B: **Identification of silverleaf whitefly resistance in pepper**. *Plant Breeding* 2011, **130**(6):708-714.

125. Coaker GL, Willard B, Kinter M, Stockinger EJ, Francis DM: **Genetic and proteomic analysis of resistance mediated by Rcm 2.0 and Rcm 5.1, two loci controlling resistance to bacterial canker of tomato**. *Phytopathology* 2005, **95**(6):S122-S122.

126. Shirasawa K, Isobe S, Hirakawa H, Asamizu E, Fukuoka H, Just D, Rothan C, Sasamoto S, Fujishiro T, Kishida Y *et al*: **SNP discovery and linkage map construction in cultivated tomato**. *DNA research : an international journal for rapid publication of reports on genes and genomes* 2010, **17**(6):381-391.

127. Consortium TTG: **The tomato genome sequence provides insights into fleshy fruit evolution**. *Nature* 2012, **485**(7400):635-641.

128. Brachi B, Morris GP, Borevitz JO: **Genome-wide association studies in plants: the missing heritability is in the field**. *Genome Biol* 2011, **12**(10).

129. Manolio TA, Collins FS, Cox NJ, Goldstein DB, Hindorff LA, Hunter DJ, McCarthy MI, Ramos EM, Cardon LR, Chakravarti A *et al*: **Finding the missing heritability of complex diseases**. *Nature* 2009, **461**(7265):747-753.

130. Banerjee S, Yandell BS, Yi N: **Bayesian Quantitative Trait Loci Mapping for Multiple Traits**. *Genetics* 2008, **179**(4):2275-2289.

131. Malosetti M, Ribaut J, Vargas M, Crossa J, Eeuwijk F: **A multi-trait multi-environment QTL mixed model with an application to drought and nitrogen stress trials in maize (Zea mays L.)**. 2008, **161**(1-2):241-257.

132. Korol AB, Ronin YI, Kirzhner VM: **Interval mapping of quantitative trait loci employing correlated trait complexes**. *Genetics* 1995, **140**(3):1137-1147.

133. deVicente MC, Tanksley SD: **QTL analysis of transgressive segregation in an interspecific tomato cross**. *Genetics* 1993, **134**(2):585-596.

134. Loren HR, Margaret AA, Robert KW: **Transgressive segregation, adaptation and speciation**. *Heredity* 1999, **83**(4):363-372.

135. Rauscher G, Simko I, Mayton H, Bonierbale M, Smart C, Grünwald N, Greenland A, Fry W: **Quantitative resistance to late blight from Solanum berthaultii cosegregates with R Pi-ber : insights in stability through isolates and environment**. *Theor Appl Genet* 2010, **121**(8):1553-1567.

136. Hospital F: **Selection in Backcross Programmes**. *Philosophical Transactions: Biological Sciences* 2005, **360**(1459):1503-1511.

137. Remington DL, O'Malley DM: **Whole-Genome Characterization of Embryonic Stage Inbreeding Depression in a Selfed Loblolly Pine Family**. *Genetics* 2000, **155**(1):337-348.

138. Brouwer DJ, St. Clair DA: **Fine mapping of three quantitative trait loci for late blight resistance in tomato using near isogenic lines (NILs) and sub-NILs**. 2004, **108**(4):628-638.

139. Giardina E, Pietrangeli I, Martone C, Zampatti S, Marsala P, Gabriele L, Ricci O, Solla G, Asili P, Arcudi G *et al*: **Whole genome amplification and real-time PCR in forensic casework**. *BMC Genomics* 2009, **10**(1):159.

140. Zhou L, Wang L, Palais R, Pryor R, Wittwer CT: **High-Resolution DNA Melting Analysis for Simultaneous Mutation Scanning and Genotyping in Solution**. *Clinical Chemistry* 2005, **51**(10):1770-1777.

141. Darvasi A, Weinreb A, Minke V, Weller JI, Soller M: **Detecting Marker-Qtl Linkage and Estimating Qtl Gene Effect and Map Location Using a Saturated Genetic-Map**. *Genetics* 1993, **134**(3):943-951.

142. Dale Young N: **A cautiously optimistic vision for marker-assisted breeding**. 1999, **5**(6):505-510.

143. Beavis WD: **The power and deceit of QTL experiments: Lessons from comparative QTL studies.** *D B Wilkinson (ed) 49th Ann Corn Sorghum Res Conf Am Seed Trade Assoc, Chicago, IL* 1994:250-266.

145. Yazdankhah SP, Kriz P, Tzanakaki G, Kremastinou J, Kalmusova J, Musilek M, Alvestad T, Jolley KA, Wilson DJ, McCarthy ND *et al*: **Distribution of Serogroups and Genotypes among Disease-Associated and Carried Isolates of Neisseria meningitidis from the Czech Republic, Greece, and Norway**. *Journal of Clinical Microbiology* 2004, **42**(11):5146-5153.

146. Maiden MCJ, Bygraves JA, Feil E, Morelli G, Russell JE, Urwin R, Zhang Q, Zhou J, Zurth K, Caugant DA *et al*: **Multilocus sequence typing: A portable approach to the identification of clones within populations of pathogenic microorganisms**. *Proceedings of the National Academy of Sciences* 1998, **95**(6):3140-3145.

147. Enright MC, Spratt BG: **Multilocus sequence typing**. *Trends in Microbiology* 1999, **7**(12):482-487.

148. Maiden MC: **Multilocus sequence typing of bacteria**. *Annual review of microbiology* 2006, **60**:561-588.

149. Jones N, Bohnsack JF, Takahashi S, Oliver KA, Chan MS, Kunst F, Glaser P, Rusniok C, Crook DW, Harding RM *et al*: **Multilocus sequence typing system for group B streptococcus**. *J Clin Microbiol* 2003, **41**(6):2530-2536.

150. Waleron M, Waleron K, Kamasa J, Przewodowski W, Lojkowska E: **Polymorphism analysis of housekeeping genes for identification and differentiation of Clavibacter michiganensis subspecies**. *European Journal of Plant Pathology* 2011, **131**(2):341-354.

151. Klement Z, Rudolph K, Sands DC: **Methods in phytobacteriology**: Akadémiai Kiadó; 1990.

152. Schaad NW, Jones JB, Chun W, Committee APSB: **Laboratory guide for identification of plant pathogenic bacteria**: APS Press; 2001.

153. Coligan JE, Kruisbeek AM, Margulies DH, Shevach EM, Strober W: **Current Protocols in Immunology**: John Wiley & Sons; 1991.

154. Tamura K, Peterson D, Peterson N, Stecher G, Nei M, Kumar S: **MEGA5: molecular evolutionary genetics analysis using maximum likelihood, evolutionary distance, and maximum parsimony methods**. *Mol Biol Evol* 2011, **28**(10):2731-2739.

155. Rozas J, Sánchez-DelBarrio JC, Messeguer X, Rozas R: **DnaSP, DNA polymorphism analyses by the coalescent and other methods**. *Bioinformatics* 2003, **19**(18):2496-2497.

156. Tajima F: **Statistical method for testing the neutral mutation hypothesis by DNA polymorphism**. *Genetics* 1989, **123**(3):585-595.

157. Feil EJ, Li BC, Aanensen DM, Hanage WP, Spratt BG: **eBURST: inferring patterns of evolutionary descent among clusters of related bacterial genotypes from multilocus sequence typing data**. *J Bacteriol* 2004, **186**(5):1518-1530.

158. Stamatakis A: **RAxML Version 8: A tool for Phylogenetic Analysis and Post-Analysis of Large Phylogenies**. *Bioinformatics* 2014.

159. Miller MA, Pfeiffer W, Schwartz T: **Creating the CIPRES Science Gateway for inference of large phylogenetic trees**. *Proceedings of the Gateway Computing Environments Workshop (GCE), 14 Nov 2010, New Orleans, LA pp 1 - 8* 2010.

160. Maddison W, Maddison D: **Mesquite: a modular system for evolutionary analysis, version 2.74**. 2010.

161. Huson DH, Bryant D: **Application of Phylogenetic Networks in Evolutionary Studies**. *Molecular Biology and Evolution* 2006, **23**(2):254-267.

162. Pritchard JK, Stephens M, Donnelly P: **Inference of Population Structure Using Multilocus Genotype Data**. *Genetics* 2000, **155**(2):945-959.

163. Hubisz MJ, Falush D, Stephens M, Pritchard JK: **Inferring weak population structure with the assistance of sample group information**. *Molecular Ecology Resources* 2009, **9**(5):1322-1332.

164. Evanno G, Regnaut S, Goudet J: **Detecting the number of clusters of individuals using the software STRUCTURE: A simulation study**. *Molecular Ecology* 2005, **14**(8):2611-2620.

165. Zaluga J, Van Vaerenbergh J, Stragier P, Maes M, De Vos P: **Genetic diversity of non-pathogenic Clavibacter strains isolated from tomato seeds**. *Systematic and applied microbiology* 2013, **36**(6):426-435.

166. Jacques MA, Durand K, Orgeur G, Balidas S, Fricot C, Bonneau S, Quillevere A, Audusseau C, Olivier V, Grimault V *et al*: **Phylogenetic analysis and polyphasic characterization of Clavibacter michiganensis strains isolated from tomato seeds reveal that nonpathogenic strains are distinct from C. michiganensis subsp. michiganensis**. *Appl Environ Microbiol* 2012, **78**(23):8388-8402.

167. Kidgell C, Reichard U, Wain J, Linz B, Torpdahl M, Dougan G, Achtman M: **Salmonella typhi, the causative agent of typhoid fever, is approximately 50,000 years old**. *Infection, Genetics and Evolution* 2002, **2**(1):39-45.

168. Spratt BG, Maiden MC: **Bacterial population genetics, evolution and epidemiology**. *Philosophical transactions of the Royal Society of London Series B, Biological sciences* 1999, **354**(1384):701-710.

169. Spratt B: **Exploring the Concept of Clonality in Bacteria**. In: *Genomics, Proteomics, and Clinical Bacteriology*. Edited by Woodford N, Johnson A, vol. 266: Humana Press; 2004: 323-352.

170. Feil EJ, Spratt BG: **Recombination and the population structures of bacterial pathogens**. *Annual review of microbiology* 2001, **55**:561-590.

171. Feil EJ, Cooper JE, Grundmann H, Robinson DA, Enright MC, Berendt T, Peacock SJ, Smith JM, Murphy M, Spratt BG *et al*: **How Clonal Is Staphylococcus aureus?** *Journal of Bacteriology* 2003, **185**(11):3307-3316.

172. Feil EJ, Maiden MC, Achtman M, Spratt BG: **The relative contributions of recombination and mutation to the divergence of clones of Neisseria meningitidis**. *Mol Biol Evol* 1999, **16**(11):1496-1502.

173. Feil EJ, Smith JM, Enright MC, Spratt BG: **Estimating recombinational parameters in Streptococcus pneumoniae from multilocus sequence typing data**. *Genetics* 2000, **154**(4):1439-1450.

174. Zanon MJ, Jorda C: **Eradication of Clavibacter michiganensis subsp michiganensis by incorporating fresh crop debris into soil: Preliminary evaluations under controlled conditions**. *Crop Protection* 2008, **27**(12):1511-1518.

175. Grogan RG, Kendrick JB: **Seed Transmission, Mode of Overwintering and Spread of Bacterial Canker of Tomato Caused by Corynebacterium-Michiganense**. *Phytopathology* 1953, **43**(9):473-473.

176. Werner NA, Fulbright DW, Podolsky R, Bell J, Hausbeck MK: **Limiting populations and spread of Clavibacter michiganensis subsp michiganensis on seedling tomatoes in the greenhouse**. *Plant Disease* 2002, **86**(5):535-542.

177. Ogawa M, Sugita S, Shimizu N, Watanabe K, Nakagawa I, Mochizuki M: **Broad-range real-time PCR assay for detection of bacterial DNA in ocular samples from infectious endophthalmitis**. 2012, **56**(6):529-535.

178. Emmatty DA, John CA: **Evaluation of Resistance of H2990, a New Tomato Cultivar, to Bacterial Canker by Different Inoculation Methods**. *Phytopathology* 1972, **62**(7):756-756.

179. Boelema BH: **Resistance to Corymebacterium michiganense in tomato cultivars and breeding lines**. *Phytophylactica* 1980, **12**:81-82.

180. Berry SZ, Madumadu GG, Uddin MR, Coplin DL: **Virulence studies and resistance to Clavibacter michiganensis ssp. michiganensis in tomato germplasm**. *Hortscience* 1989, **24**(2):362-365.

181. Altmüller J, Seidel C, Lee Y-A, Loesgen S, Bulle D, Friedrichs F, Jellouschek H, Kelber J, Keller A, Schuster A *et al*: **Phenotypic and genetic heterogeneity in a genome-wide linkage study of asthma families**. *BMC Pulm Med* 2005, **5**(1):1-10.

182. Brouwer DJ, St. Clair DA: **Fine mapping of three quantitative trait loci for late blight resistance in tomato using near isogenic lines (NILs) and sub-NILs**. *Theor Appl Genet* 2004, **108**(4):628-638.

183. Monforte AJ, Tanksley SD: **Fine mapping of a quantitative trait locus (QTL) from Lycopersicon hirsutum chromosome 1 affecting fruit characteristics and agronomic traits: breaking linkage among QTLs affecting different traits and dissection of heterosis for yield**. *Theor Appl Genet* 2000, **100**(3-4):471-479.

184. Bernacchi D, Tanksley SD: **An interspecific backcross of Lycopersicon esculentum x L. hirsutum: linkage analysis and a QTL study of sexual compatibility factors and floral traits**. *Genetics* 1997, **147**(2):861-877.

185. Brachi B, Faure N, Bergelson J, Cuguen J, Roux F: **Genome-wide association mapping of flowering time in in nature: genetics for underlying components and reaction norms across two successive years**. *Acta botanica Gallica : bulletin de la Societe botanique de France* 2013, **160**(3-4):205-219.

186. Charlesworth D, Willis JH: **The genetics of inbreeding depression**. *Nat Rev Genet* 2009, **10**(11):783-796.

187. Keller LF, Waller DM: **Inbreeding effects in wild populations**. *Trends in Ecology & Evolution*, **17**(5):230-241.

188. Burokienė D, Sobiczewski P, Berczyn´ski S: **Phenotypic characterization of Clavibacter michiganensis subsp. michiganensis isolates from Lithuania**. *Phytopathologia Polonica* 2005, **38** 63-77.

189. Louws FJ, Bell J, Medina-Mora CM, Smart CD, Opgenorth D, Ishimaru CA, Hausbeck MK, de Bruijn FJ, Fulbright DW: **rep-PCR-mediated genomic fingerprinting: A rapid and effective method to identify Clavibacter michiganensis**. *Phytopathology* 1998, **88**(8):862-868.

190. Jong J, Honma S: **Evaluation of screening techniques and determination of criteria for assessing resistance to Corynebacterium michiganense in tomato**. *Euphytica* 1976, **25**(1):405-414.

191. van Loon LC, Geraats BP, Linthorst HJ: **Ethylene as a modulator of disease resistance in plants**. *Trends Plant Sci* 2006, **11**(4):184-191.

192. Groen SC, Whiteman NK, Bahrami AK, Wilczek AM, Cui J, Russell JA, Cibrian-Jaramillo A, Butler IA, Rana JD, Huang GH *et al*: **Pathogen-triggered ethylene signaling mediates systemic-induced susceptibility to herbivory in Arabidopsis**. *Plant Cell* 2013, **25**(11):4755-4766.

193. Mantelin S, Bhattarai KK, Kaloshian I: **Ethylene contributes to potato aphid susceptibility in a compatible tomato host**. *The New phytologist* 2009, **183**(2):444-456.

194. Cohn JR, Martin GB: **Pseudomonas syringae pv. tomato type III effectors AvrPto and AvrPtoB promote ethylene-dependent cell death in tomato**. *The Plant Journal* 2005, **44**(1):139-154.

195. Taga ME, Bassler BL: **Chemical Communication among Bacteria**. *Proceedings of the National Academy of Sciences of the United States of America* 2003, **100**:14549-14554.

196. Waters CM, Bassler BL: **QUORUM SENSING: Cell-to-Cell Communication in Bacteria**. *Annual Review of Cell and Developmental Biology* 2005, **21**(1):319-346.

197. Garg N, Manchanda G, Kumar A: **Bacterial quorum sensing: circuits and applications**. *Antonie van Leeuwenhoek* 2014, **105**(2):289-305.

198. Novick RP, Geisinger E: **Quorum Sensing in Staphylococci**. *Annu Rev Genet* 2008, **42**(1):541-564.

199. Jansen RC, Nap JP: **Genetical genomics: the added value from segregation**. *Trends in genetics : TIG* 2001, **17**(7):388-391.

200. Juenger TE, Wayne T, Boles S, Symonds VV, McKay J, Coughlan SJ: **Natural genetic variation in whole-genome expression in Arabidopsis thaliana: the impact of physiological QTL introgression**. *Molecular Ecology* 2006, **15**(5):1351-1365.

201. Cai G, Restrepo S, Myers K, Zuluaga P, Danies G, Smart C, Fry W: **Gene profiling in partially resistant and susceptible near-isogenic tomatoes in response to late blight in the field**. *Molecular plant pathology* 2013, **14**(2):171-184.

202. Lin F, Zhao M, Baumann D, Ping J, Sun L, Liu Y, Zhang B, Tang Z, Hughes E, Doerge R *et al*: **Molecular response to the pathogen Phytophthora sojae among ten soybean near isogenic lines revealed by comparative transcriptomics**. *BMC Genomics* 2014, **15**(1):18.

203. Broekaert WF, Delaure SL, De Bolle MF, Cammue BP: **The role of ethylene in host-pathogen interactions**. *Annual review of phytopathology* 2006, **44**:393-416.

204. Chen X, Niks R, Hedley P, Morris J, Druka A, Marcel T, Vels A, Waugh R: **Differential gene expression in nearly isogenic lines with QTL for partial resistance to Puccinia hordei in barley**. *BMC Genomics* 2010, **11**(1):1-13.

205. Gilliland LU, Magallanes-Lundback M, Hemming C, Supplee A, Koornneef M, Bentsink L, DellaPenna D: **Genetic basis for natural variation in seed vitamin E levels in Arabidopsis thaliana**. *Proceedings of the National Academy of Sciences* 2006, **103**(49):18834-18841.

206. Keurentjes JJ: **Genetical metabolomics: closing in on phenotypes**. *Curr Opin Plant Biol* 2009, **12**(2):223-230.

207. Dunaevsky YE, Elpidina EN, Vinokurov KS, Belozersky MA: **Protease Inhibitors in Improvement of Plant Resistance to Pathogens and Insects**. *Mol Biol* 2005, **39**(4):608-613.

208. Ryan CA: **Protease Inhibitors in Plants: Genes for Improving Defenses Against Insects and Pathogens**. *Annual review of phytopathology* 1990, **28**(1):425-449.

209. Khadeeva NV, Kochieva EZ, Tcherednitchenko MY, Yakovleva EY, Sydoruk KV, Bogush VG, Dunaevsky YE, Belozersky MA: **Use of buckwheat seed protease inhibitor gene for improvement of tobacco and potato plant resistance to biotic stress**. *Biochemistry Biokhimiia* 2009, **74**(3):260-267.

210. Kalia VC, Purohit HJ: **Quenching the quorum sensing system: potential antibacterial drug targets**. *Critical Reviews in Microbiology* 2011, **37**(2):121-140.

211. **GM crops: A story in numbers**. *Nature* 2013, **497**(7447):22-23.

Summary

Clavibacter michiganensis subsp. michiganensis (*Cmm*) is the pathogen causing bacterial canker in tomato. The disease was described for the first time in 1910 in Michigan, USA. *Cmm* is considered the most harmful bacteria threatening tomato growth worldwide. Disease transmission occurs via seed and symptoms become visible at least 20 days after infection. Due to its complex strategy and transmission, *Cmm* is under quarantine regulation in EU and other countries. There is no method to stop disease progress in plants after infection. Thus, disease management consists usually of chemical treatments as protection and by careful clean cultural practices. However, the use of resistant varieties is the most effective and environmentally friendly method. Unfortunately, there is no cultivar harboring effective resistance on the market although efforts to get resistant varieties already started in the 60s. Our aim of the work described in this thesis was to develop valuable genetic material for breeders in order to enable them to release resistant cultivars in the future and provide comprehensive scientific knowledge for further detailed research about *Cmm*.

Our scientific activity described in this thesis started with the identification of new *Cmm* resistance sources and confirmation of existing ones. In Chapter 3 we describe the results of screening a collection of wild tomatoes for resistance to *Cmm*. We made use of Real Time TaqMan PCR for intensive phenotyping. Using wilting and bacterial concentration as parameters for evaluation of wild genotypes, we have identified new sources and confirmed existing ones. We decided to continue further with one new source, *S. pimpinellifolium*, and one already known existing source, *S . arcanum.*

We continued our research in Chapter 4 with a genetic analysis of the new source coming from *S. pimpinellifolium*. A recombinant inbred line population between the resistant parent, *S. pimpinellifolium*, and the susceptible parent *S. lycopersicum* was evaluated in three different environments. Wilting, bacterial concentration, and stem discoloration were the scored parameters. Responses of resistance in different environments were determined and genomic regions responsible for different responses were mapped.

In Chapter 5, we describe the results of our research on fine mapping of previously identified genomic regions and developing nearly isogenic lines containing those genomic regions. For fine mapping, we made use of old stock DNA and recently developed different types of SNP marker technology. Previously identified Quantitative Trait Loci (QTL) could be more precisely delimited. During isogenic line development, embryo rescue was used in order to break the genetic barrier between our *S. arcanum* source and tomato. Marker assisted backcrossing was applied to obtain lines with a minimum of donor parent in a faster way. By using this method we gained two generations of backcrossing.

In order to obtain comprehensive information about different *Cmm* isolates in Turkey, we have performed multi locus sequence analysis (MLST) analysis on a *Cmm* collection, which was collected in 20 years in different parts of Turkey. In Chapter 6 a statistical analysis of this

collection revealed that measurement of clonality of this collection was possible as well as it was possible to predict the virulence level of strains using a subset of housekeeping genes.

All knowledge gained by our experiments and knowledge coming from literature about *Cmm* have led to a review paper (Chapter 2), in which comprehensive information about *Cmm* resistance sources, genetic analysis of these sources, detection methods of *Cmm*, infection strategies of *Cmm* and interaction with its host was discussed.

In conclusion, two good *Cmm* resistance sources and advanced material and methods have now become available for breeders. Genomic regions of these sources associated with resistance were determined. Wider knowledge about *Cmm* detection, *Cmm* infection and *Cmm* interaction with its host are available for future research.

Samenvatting

Clavibacter michiganensis subsp. *michiganensis (Cmm)* is de ziekteverwekker die de verwelkingsziekte in tomaat veroorzaakt. Deze ziekte is voor het eerst beschreven in 1910 in Michigan, USA. *Cmm* wordt wereldwijd beschouwd als de meest schadelijke bacterieziekte in tomaat. Verspreiding van de ziekteverwekker vindt plaats via zaad en de eerste symptomen worden 20 dagen na infectie zichtbaar. Door zijn complexe infectiestrategie en verspreiding, valt *Cmm* onder quarantaine regelingen van de Europese Unie en andere landen. Er is geen methode om de ziekteverspreiding tegen te gaan nadat de plant eenmaal geïnfecteerd is. Dientengevolge is het vooral belangrijk infectie te voorkomen middels chemische behandelingen en zorgvuldig schoon werken. Het gebruik van resistente rassen is een effectief en omgevingsvriendelijke methode. Jammer genoeg is er nog geen tomatenras beschikbaar met een resistentie die voldoende effectief is, ondanks dat de pogingen om resistente rassen te krijgen al in de zestiger jaren begonnen zijn. Ons doel van het werk, beschreven in dit proefschrift, was waardevol genetisch materiaal te ontwikkelen om veredelaars in staat te stellen in de toekomst resistente rassen te verkrijgen en meer wetenschappelijk kennis te vergaren die verder gedetailleerd onderzoek mogelijk maken.

Ons wetenschappelijk werk begon met de identificatie van nieuwe *Cmm* resistentiebronnen en de bevestiging van resistentie in eerder gevonden bronnen. In Hoofdstuk 3 beschrijven we de resultaten van een toetsing van een verzameling wilde tomaten op resistentie tegen *Cmm*. De parameters verwelking en bacterieconcentratie werden hiervoor gebruikt. We maakten gebruik van Real Time Taqman PCR voor het bepalen van bacterieconcentraties. We besloten verder te gaan met een nieuwe bron, *S. pimpinellifolium,* en een reeds bekende bron, *S. arcanum*.

Ons onderzoek, zoals beschreven in Hoofdstuk 4, ging verder met een genetische analyse van de nieuwe bron, een accessie van *S. pimpinellifolium*. Een recombinant inteeltlijnpopulatie, ontwikkeld na een kruising tussen de resistente ouder, *S. pimpinellifolium*, en een vatbare ouder *S. lycopersicum*, is geëvalueerd onder drie verschillende omstandigheden. Verwelking, bacterieconcentratie, en stengelverkleuring waren de gebruikte parameters. De resistentieniveaus zijn bepaald onder de verschillende omstandigheden en gebieden op het genoom geassocieerd met een verhoogde resistentie zijn geïdentificeerd.

In Hoofdstuk 5 beschrijven we het kleiner maken van eerder geïdentificeerde genomische gebieden en het maken van bijna isogene lijnen. Voor het fijnkarteren konden we gebruik maken van twintig jaar oud DNA in combinatie met recentelijk ontwikkelde markertechnologie. Op deze wijze konden eerder geïdentificeerde Quantitative Trait Loci (QTLs) nauwkeuriger begrensd worden. Om isogene lijnen te ontwikkelen was het nodig de embryo's te redden en zo de genetische barrières tussen *S. arcanum* en *S. lycopersicum* te doorbreken. Merker gestuurde

terugkruisingen zijn gebruikt om die planten te kiezen met een minimum aan genetisch materiaal van de *S. arcanum* donor te verkrijgen. Met behulp hiervan was het mogelijk het terugkruisen met twee generaties te reduceren.

Om uitgebreide informatie te verkrijgen over de verschillende *Cmm* isolaten in Turkije hebben we een multi locus sequentie analyse (MLST) analyse van een *Cmm* collectie uitgevoerd. Deze collectie was verzameld gedurende 20 jaar in verschillende gebieden van Turkije. In Hoofdstuk 6 laat een statistische analyse zien dat de klonaliteit van deze collectie bepaald kon worden en dat het mogelijk was het virulentieniveau van de isolaten te voorspellen met behulp van de sequentie van een set van huishoudgenen.

Alle kennis uit onze experimenten over *Cmm* en de beschikbare informatie hebben geleid tot een overzichtsartikel (Hoofdstuk 2), waarin uitgebreide informatie over *Cmm* resistentiebronnen, genetische analyses, detectiemethodes, infectiestrategieën en de interactie van *Cmm* met zijn gastheer beschreven en bediscussieerd worden.

De conclusie van het beschreven werk is dat er twee goede resistentiebronnen gevonden zijn en dat er verscheidene materiaal en methoden beschikbaar zijn gekomen voor veredelaars. Meer kennis over *Cmm* detectie, infectie en interactie met de gastheer zijn nu beschikbaar voor verder onderzoek.

Acknowledgements

Wageningen was the seventh and the final stop of 22 years of my study life. The city is small but the content is big. We call it an international village where you can find many people having different nationalities from all over the world. My journey in this final stop has started in 2008 when I asked Prof. Richard Visser for a PhD position at the department of Plant Breeding of Wageningen University. I was accepted for the PhD program with a condition of extra financial support. Therefore, I would like to give many thanks to Prof. Richard Visser for accepting me as a PhD candidate, for his valuable contributions for our PhD project and for the publications yielded from this project. However, it is my privilege to express my special thanks and sincere appreciations to Dr. Sjaak van Heusden for accepting to supervise me in whole 5 years of my PhD study. Sometimes we had some difficulties but most of the time it was fun and pleasure for me to work with him. He was always cheerful which made my day better and he was always open for new ideas and methods. Thanks to him for supporting me whenever I asked about taking PhD courses and attending international congress. Although most of the time he traveled all around the world it was still possible to be supervised by him even by emails.

I should send my special thanks to my external supervisor Dr. Jan der Wolf and his team, Patricia van der Zouwen and Marjon Krijger, from Plant Research International Biointeractions and Plant Health group, who helped me to develop a running protocol for quantification of bacteria.

I would like to thank many people at Plant Breeding Department for their scientific help and friendship. Thanks to Dr. Chris Maliepaard with whom I had many discussions on statistics and shared a friendly environment near the coffee machine. I also would like to thank to Dr. Geert Jan de Klerk who helped me to find my way in medium selection and with whom I shared many discussions. Also many thanks to Fien and Bernadette for guiding me in the laboratory work.

My friends at Plant Breeding, I am very grateful to you all. It was wonderful to share moments near the coffee machine with you and at corridors or in the labs or even in De Zaaijer. My roommates Ram, Xi, Efstathios, Maria and Aziz , it was lovely to share working room with you with respectful and very friendly environment. Also Arwa and Mirjana, it was nice to steal your chocolates from your desk and having many funny discussions with you and Alex. A lot of thanks to many other friends, Alejandro, Thijs, Christos, Pierre, Paula, Peter, Henk Schouten, Eric van de Weg, Yolanda, Rafael and Marcella, Johan, Arnaud Bovy, Koen, Syrafin, Awang, Antoine, Nasim, Gullia and many more. Also, I need to mention students and many thanks to them, Feng, Jane, Henri, Duygu and Rudil, who did their MSc thesis or internship with me and made contributions to the project.

I should also mention to the Wageningen Turkish team with whom I have organized many parties and dinners. Gamze, Onur, Oylum, Rozelin, Şafak, Morteza, Yunus, Mustafa, Sevinç, Başak, Sami Alev-Alper, Dilek-Raimon and so many other I do not remember the names. It was really relaxing and enjoyable to have many lovely foods, discussions and travels with you.

And of course many international friends in an international village. Alexandra, Robin, Maria, Miriam, Pascal, Olga, Slava, Anna Pukinova and many others, thanks to all my dears for making life colorful in this small village. I should not forget to mention my Salsa friends in ISOW with whom i enjoyed the many moments and had nice dance evenings. Another thanks should go to my Bongerd Boxing team for the nice training environment of 3 years.

I should also thank to Dersim community in The Nerherlands. Hüseyin, Ali, Eylem and Haydar Bra, haq razi vo sımare. Seweta sıma ez zone xu tenena muso.

Special thanks to my paranymphs Gert van Arkel and Marian Oortwijn to share this special moment with me.

Finally, my most special thanks to my family. They always encourage me and trust me. With their love I am always very strong in life. Haq sımare raji vo. Xızır le sıma de vo. Sevgili ailem, her zaman beni desteklediğiniz ve güvendiğiniz için size sonsuz sevgi ve teşekkürler.

Yusuf ŞEN

Wageningen University

Yusuf ŞEN was born on January 1st, 1981 in Tunceli city of Turkey. He studied at Akdeniz University at Faculty of Agriculture with specialization on Soil Science and graduated in 2005. Right after he attended MSc study at Warsaw University of Life Sciences at Plant Physiology Department with specialization on Seed Priming. After more than a year working in agriculture sector, he started his PhD study in January 2009 upon acceptance to Wageningen University Department of Plant Breeding. He obtained a scholarship for the first two years from The Scientific and Technological Research Council of Turkey (TÜBİTAK) to pursue his PhD study.

Education Statement of the Graduate School

Experimental Plant Sciences

The Graduate School EXPERIMENTAL PLANT SCIENCES

Issued to: Yusuf Sen
Date: 29 September 2014
Group (s): Plant Breeding
University: Wageningen University & Research Centre

1) Start-up phase	*date*
► **First presentation of your project**	
Breeding for resistance to bacterial canker	Apr 21, 2009
► **Writing or rewriting a project proposal**	
Bacterial canker of tomato: current knowledge of detection, management, resistance and interactions, to be published in Plant Science, *accepted for publication*, 2014	Aug 2014
► **MSc courses**	
GEN-30306: Genetic Analyisis Tools and Concept (GATC)	Dec 31, 2009
► **Laboratory use of isotopes**	
Subtotal Start-up Phase	*13.5 credits**

2) Scientific Exposure	*date*
► **EPS PhD student days**	
EPS PhD student days, Leiden University	Feb 29, 2009
EPS PhD student day, Utrecht University	Jun 01, 2010
EPS PhD student day, Wageningen University	May 20, 2011
EPS PhD student day, University of Amsterdam	Nov 30, 2012
► **EPS theme symposia**	
EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents', University of Amsterdam	Feb 03, 2011
EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents', Wageningen University	Feb 10, 2012
EPS Theme 2 symposium 'Interactions between Plants and Biotic Agents', Utrecht University	Jan 24, 2013
► **NWO Lunteren days and other National Platforms**	
ALW meeting `Experimental Plant Sciences`Lunteren, NL	Apr 19-20, 2010
ALW meeting `Experimental Plant Sciences`Lunteren, NL	Apr 04-05, 2011
ALW meeting `Experimental Plant Sciences`Lunteren, NL	Apr 02-03, 2012
► **Seminars (series), workshops and symposia**	
Invited seminar Theo van der Lee, 'Pathoscreen and its application in resistance phenotyping'	Feb 21, 2011
Plant Sciences seminar 'High throughput plant phenotyping (HTPP), a rapidly growing activity'	May 10, 2011
Invited seminar Veronica Grieneisen	Nov 17, 2011
ExPectationS day (EPS Career Day), Wageningen	Nov 18, 2011
Symposium 'Plant Breeding in the Genomics Era', Wageningen	Nov 25, 2011
WEES seminar Marc van Roosmalen	Dec 07, 2011
Plant Sciences seminar 'An interactive presentation on Open Science'	Dec 13, 2011
Invited seminar Graham Seymour, 'The Tomato Genome: From Genes To QTL and Networks'	Jan 24, 2012
Symposium 'Improving yield prediction by combining statistics, genetics,physiology and phenotyping: the EU SPICY project in pepper'	Mar 07-09, 2012
Invited seminar Hong Ma, 'Molecular genetic, transcriptomic and genomic characterization of meiotic recombination in Arabidopsis'	May 29, 2012
Invited seminar Salvatore Ceccarelli 'Participatory Plant Breeding - a response to the problems of hunger, biodiversity and climate changes'	May 29, 2012
Invited seminar Patrick Forterre, 'New concepts on the origin and nature of viruses: their major role in both ancient and recent biological evolution'	Oct 18, 2012
Symposium 'Itraspecific Pathogen Variation - Implications and Opportunities', Wageningen	Jan 22, 2013
► **International symposia and congresses**	
Eucarpia tomato, Malaga (Spain)	Mar 11-14, 2011
Biotechnology and other omics in Vegetable Science, Antalya (Turkey)	Apr 29-May 02, 2012
8th Solanaceae and 2nd Cucurbitaceae Genome Joint Conference, Kobe (Japan)	Nov 28-Dec 02, 2011
International Plant Breeding Conference, Antalya (Turkey)	Nov 11-14, 2012
Next Generation Plant Breeding, Ede (The Netherlands)	Nov 10-14, 2013
► **Presentations**	
poster: Eucarpia tomato, Malaga, Spain	Mar 11-14, 2011
poster: ExPectationS day (EPS Career day)	Nov 18, 2011
poster: 8th Solanaceae and 2nd Cucurbitaceae Genome Joint Conference, Kobe (Japan)	Nov 28-Dec 02, 2011
Oral: Biotechnology and other omics in Vegetable Science, Antalya (Turkey)	Apr 29-May 02, 2012
► **IAB interview**	
Meeting with a member of the International Advisory Board of EPS	Feb 17, 2011
► **Excursions**	
Visit 3 seed companies, Hortifair-Amsterdam (2009 and 2011), KeyGene 26 January 2012	2009, 2011, 2012
Subtotal Scientific Exposure	*17.2 credits**

3) In-Depth Studies		*date*
►	**EPS courses or other PhD courses**	
	Utrecht Summerschool Environmental Signaling	Aug 24-26, 2009
	Plant Metabolomics	Mar 26-28, 2011
	Basic Statistic	Jun 22-29, 2011
	Statistical learning methods for DNA-based prediction of complex traits	Oct 17-21, 2011
	Generalized linear Models	Jun 14-15, 2012
	Mixed Linear Models	Jun 21-22, 2012
	Mixed model based QTL mapping in GenStat	Jul 14-16, 2012
	Bioinformatic: a user approach	Aug 27-31, 2012
	Current Trends in Phylogenetics	Oct 22-26, 2012
►	**Journal club**	
	Member of literature discussion group at Plant Breeding	2009 - 2013
►	**Individual research training**	
	Subtotal In-Depth Studies	*12.9 credits**

4) Personal development		*date*
►	**Skill training courses**	
	Techniques for writing and presenting a scientific paper	Oct 19-22, 2010
	Advance statistic course: Design of Experiments	Oct 12-14, 2011
	Introduction to R	Nov 17-18, 2011
►	**Organisation of PhD students day, course or conference**	
►	**Membership of Board, Committee or PhD council**	
	Subtotal Personal Development	*2.8 credits**

TOTAL NUMBER OF CREDIT POINTS*	**46.4**
Herewith the Graduate School declares that the PhD candidate has complied with the educational requirements set by the Educational Committee of EPS which comprises of a minimum total of 30 ECTS	
** A credit represents a normative study load of 28 hours of study.*	

The research described in this thesis was financially supported by Wageningen UR Plant Breeding and The Scientific and Technological Research Council of Turkey (TÜBİTAK).

Printed by Ipskamp Drukkers Nijmegen, The Netherlands