CHARACTERIZATION OF ATYPICAL *CLAVIBACTER MICHIGANENSIS* subsp. *MICHIGANENSIS* POPULATIONS IN GREENHOUSE TOMATOES IN ITALY

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SUMMARY

The quarantine bacterium *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*) is the agent of tomato bacterial canker (TBC), one of the most destructive bacterial diseases of this crop, that causes severe economic losses worldwide. During a recent outbreak of TBC in Sicily (insular Italy), it was impossible to amplify the *pat*-1 gene by PCR with CMM5/CMM6 primers from affected greenhouse-grown plants from three farms, although *Cmm*-like colonies were consistently isolated. Microlog metabolic profile, 16S rDNA sequencing and the positive amplification of other *Cmm* DNA targets supported the identification of the strains as *Cmm*, suggesting the occurrence of a population lacking the virulence gene *pat*-1. A detailed phenotypic and molecular characterization of these *Cmm* strains and their virulence to tomatoes, as compared with *Cmm* reference strains is reported in this study. Pathogenicity tests revealed the strains under study were able to induce TBC, although delayed wilting symptoms were observed. Strains with or without *pat*-1 were undistinguishable, based on disease indexes.

Key words: Tomato bacterial canker, atypical strains, virulence, PCR, pathogenicity tests.

INTRODUCTION

In recent years an increasing number of outbreaks of tomato bacterial canker (TBC), one of the most destructive bacterial diseases of tomato causing severe economic losses worldwide have been reported (de Leon *et al.,* 2011). The causal agent of TBC is *Clavibacter michiganensis* subsp. *michiganensis* (*Cmm*), which is regulated as a harmful organism for the EU (Annex II A section II), and has been detected in most of the countries of the European and Mediterranean Plant Protection Organization (EPPO). It is included in the A2 list by the EPPO, the Asia and Pacific Plant Protection Commission (APPPC), the Caribbean Plant Protection Commission (CPPC) and the Inter-African Phytosanitary Council (IAPSC) (de Leon *et al.*, 2011).

Infected seed is the primary source of infection (Thyr *et al.*, 1973; Gitaitis *et al.*, 1992). *Cmm* enters tomato plants through wounds on roots or stems from where it reaches the xylem vessels inducing systemic infections (Meletzus *et al.*, 1993). Later, cellulase and other plant cell wall hydrolyzing extracellular enzymes provide sugars as nutrients for the bacterium (Gartemann *et al.*, 2008). Symptoms begin mainly as unilateral wilt of the leaves that later spread to the whole foliage, cankers develop on the stems and in an advanced stage of infection the plants die. Secondary infections derive from masses of bacteria oozing through cracks at the plant surface, can take place through broken trichomes on the leaves, wounds or hydathodes (de Leon *et al.*, 2011). Conflicting experimental trials showed pathogen survival in soil, in infected debris for a variable time, depending on the geographic location, soil type, moisture, temperature and detection method (Bryan, 1930; Strider, 1967; Echandi, 1971; Gleason *et al.*, 1991; Basu, 1970; Moffet *et al.*, 1984). *Cmm* can survive in different solanaceous and non-solanaceous plant species (de Leon *et al.*, 2011).

Two major pathogenicity genes, *pat*-1 and *celA*, were identified on two circular plasmids in the bacterial strain NCPPB 382 (Meletzus *et al.*, 1993; Dreier *et al.*, 1997). The *pat*-1 gene, localized on plasmid pCM2 (70.0 kbp), encodes a protein of 280 amino acids with a molecular mass of 29.7 kDa (Dreier *et al.*, 1997). Pat-1 protein exhibits significant homology to several serine proteases of the trypsin type (Stork *et al.*, 2008). It is a member of the serine peptidase family S1A. Pat-1 and related serine peptidases of *Cmm* form a family, designated Chp family, one of the three different families of *Cmm* extracellular serine peptidases (Gartemann *et al.*, 2008). The *celA* gene on plasmid pCM1 (27.4 kbp) encodes a β-1,4-endoglucanase (Jahr *et al.*, 2000). CelA consists of three domains. The third expansin-like domain is essential both for the development of wilting symptoms and degradation of crystalline cellulose (Jahr *et al.*, 2000). The two genes are indispensable for full virulence expression of the bacterium *in planta*. Indeed,

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a plasmid-free derivative strain of NCPPB 382, designated CMM100, obtained by curing of the plasmids from wild type strain with high temperature, colonizes tomato plants efficiently, produces EPS, but does not induce disease on tomato (Meletzus and Eichenlaub, 1991). Cured bacterial strains harbouring only one plasmid, CMM101 (pCM1) and CMM102 (pCM2), showed reduced virulence (Meletzus *et al.*, 1993). A putative pathogenicity island (PAI; *chp/tomA*) with a low G+C content necessary for pathogenicity and colonization of tomato was also identified in the NCPPB 382 genome (Gartemann *et al.*, 2008). The plasmid-borne *celA* and *pat*-1 genes are strongly induced during the early stage of infection *in planta* but their expression is reduced in a mutant lacking the putative PAI of NCPPB 382 (Chalupowicz *et al.*, 2010).

During 2007 from greenhouses of three farms located in one of the most important Italian greenhouse tomato production area (Ragusa province, insular Italy) the presumptive diagnosis of TBC-affected tomatoes based on PCR with CMM5/CMM6 primers (amplifying a fragment of the *pat*-1 gene), failed although *Cmm*-like colonies were consistently isolated. These colonies were also negative by PCR, suggesting the presence of a population lacking the target gene. The aim of the present study was to perform a phenotypic and molecular characterization of these *Cmm* strains and to investigate their virulence on tomato plants compared to *Cmm* reference strains.

MATERIALS AND METHODS

Isolation procedure and phenotypic characterization of *C. michiganensis* subsp. *michiganensis* strains. Isolation of *Cmm* was attempted from symptomatic tomato plants collected from three greenhouses of different farms located in the Ragusa province. Small sections of affected vascular tissue were removed, ground in a small volume of sterile distilled water and plated on nutrient agar supplemented with 1% D-glucose (NGA) (OEPP/EPPO, 2005). Presumptive colonies of the pathogen were purified by restreaking twice on NGA. The bacteria obtained were tested for the following traits: Gram stain, metabolism of glucose, aesculin, starch and casein hydrolysis, oxidase and catalase activities (Schaad *et al.*, 2001; OEPP/EPPO, 2005). Representative strains were further characterized by metabolic profiling using the Biolog MicroLog3™ Microbial Identification system (Biolog, USA) following the manufacturer's instructions. After 16-24 h incubation at 30°C plates were read in a Biolog Microstation microplate reader (Bio-Tek Instruments, USA). *Cmm* strain NCPPB 382, its plasmid-free derivative CMM100 and three *Cmm* strains from our collection isolated in the same tomato-production area although from different farms were used as references (Table 1). All strains were routinely maintained on NGA at 4°C for short periods and preserved in 15% glycerol at -80°C for long-term storage.

Preparation of bacterial and plant DNA. Target bacterial DNAs for PCR amplification were either: (i) cell lysates obtained by heat lysis of a bacterial suspension containing a single *Cmm* colony from a 72-hour-old culture in 100 µl of distilled sterile water (OEPP/EPPO, 2005) or (ii) pure DNA extracted from 1 ml of bacterial cultures grown in Nutrient Broth on a rotatory shaker for 48 h at 27°C using the Puregene Yeast/ Bacteria Kit (Qiagen, Italy), according to the manufacturer's instructions.

Target DNA from plant samples was obtained from small pieces of infected stems as follows: (i) maceration in sterile distilled water for 5-10 min (EPPO/OEPP, 2005), (ii) grinding small sections of discoloured vascular tissue in sterile distilled water, or (iii) extraction of total DNA by the Puregene Yeast/Bacteria Kit (Qiagen, Italy). Either stem portions of tomato plants artificially inoculated with *Cmm* NCPPB 382 or tomato plant extracts additioned with an aqueous suspension of the same strain to a final concentration of 10^6 CFU/ml were used as positive controls.

PCR analysis. Four sets of oligonucleotides were used: CMM5/CMM6 amplifying a part of the *pat*-1 gene located on the pCM2 plasmid and generating a 614 bp PCR amplification product (Dreier *et al.*, 1995); P1rep/P3rep, amplifying a 219 bp DNA fragment of the repetitive *pat-1-rep* motif located downstream of *pat*-1 (Dreier *et al.,* 1997); PFC3/PFC5, amplifying a 552 bp subgenic fragment of the *celA* localized in plasmid pCM1 (Kleitman *et al.,* 2008); PSA-4/PSA-R, derived from the 16S-23S rRNA intergenic spacer region and amplifying an amplicon of 270 bp (Pastrik and Rainey, 1999). The reaction mixtures (25 µl) contained 1X PCR buffer (Invitrogen, USA), 100 µM (PSA-4/PSA-R) or 200 µM (P1rep/P3rep; CMM5/CMM6; PFC3/PFC5) of each dNTP (Invitrogen, USA), 0.2 µM (PSA-4/PSA-R), 0.4 µM (PFC3/PFC5) and 0.8µM (CMM5/CMM6; P1rep/P3rep) of each primer, 1 U *Taq* DNA polymerase (Invitrogen, USA). One microliter of DNA or 2.5 µl of cell lysate were used as template. PCR cycling was performed using the conditions previously reported (Dreier *et al.*, 1995, 1997; Pastrik and Rainey, 1999; Kleitman *et al.*, 2008).

All PCR reactions were carried out in a GeneAmp 9700 thermocycler PCR system (PE Applied Biosystem, USA). The amplificates were subjected to electrophoresis on 1% agarose gels, and were stained with SYBR Safe DNA gel stain (Invitrogen, USA). Bacteria and plant samples negative in PCR assays were retested twice.

16S rDNA sequence analysis. 16S rDNA PCR from

representative strains was conducted using the universal primers 530F/Uni1492 (Lane, 1991). PCR reactions were carried out in a total reaction volume of 50 µl. Reaction mixes contained the following Invitrogen (USA) products: 5 µl of 10X PCR buffer, 1.5 mM $MgCl_2$, 200 µM dNTPs, 0.2 µM each primer (MWG), and 2.5 units of *Taq* DNA polymerase. Cycling conditions consisted of 5 min of initial melting at 95°C, followed by 35 cycles of 1 min melting at 94°C, 1 min annealing at 50°C, and 2 min extension at 72°C. These cycles were followed by a final 10 min elongation at 72°C. Amplicons were purified using the QiaQuick Gel Extraction Kit (Qiagen, Italy) following the manufacturer's instructions and quantified. 16S rDNA sequences were determined at the Bio Molecular Research Center (BMR), University of Padua, Italy. The BLASTn program (Madden *et al.*, 1996, http://www.ncbi.nlm.nih.gov/blast) was used to find sequences nearly identical to the 16S rDNA fragments sequenced in this work, which are deposited in GenBank under the accession Nos JQ917461 and JQ917462.

BOX-PCR fingerprinting. Genomic fingerprinting of *Cmm* strains was carried out using primers corresponding to the BOX element (Versalovic *et al.,* 1994). The reactions were conducted according to De Bruijn *et al.* (1992). Briefly, approximately 100 ng of purified DNA was used as a template in a 25 µl reaction mixture containing 1X Gitschier buffer, 20 mg/ml BSA, 2.5 mM of each dNTP, 2.5 µM of the primer Box A1R, 2.5 µl of DMSO, 1 U of Platinum *Taq* DNA polymerase (Invitrogen, USA). Amplification was performed in an Thermal cycler (Perkin Elmer 9700) with the following program: an initial denaturation step of 5 min at 95°C, followed by 30 cycles of 30 sec at 94°C, 1 min at 53°C and 8 min at 65°C with a final elongation step of 15 min at 65°C. BOX-PCR products were separated by electrophoresis in 2% agarose gel in TAE buffer at 5.6 V/cm^{-1} for 2.5 h. The GeneRuler™ 1Kb DNA ladder (Fermentas, Lithuania) was included as molecular weight marker. Gels were stained with SYBR Safe (Invitrogen, USA) and photographed under UV light.

Pathogenicity tests. Pathogenicity tests were carried out on tomato plants cv. Moneymaker by leaf clip and root dip methods. Trials were performed in growth chambers at 25°C with 12/8 h day/night photoperiod.

For leaf clip inoculations, tomato seedlings were grown in soil-filled trays and the first true leaf of 4 week-old-plants was cut with a scalpel contaminated with *Cmm* cells scraped from a 48 h NGA culture. A non-contaminated sterile scalpel was used for inoculating control plants. Thirty tomato plants per strain were inoculated. Plants were maintained under high humidity and were observed daily and rated for symptoms 8, 14, and 21 days post inoculation (dpi) using an additive disease rating scale of 0 to 5 (Francis *et al.*, 2001). A score of 0 indicated no symptoms and one point was assigned for typical symptoms of TBC: marginal necrosis, wilt and canker. A half-point was deducted if symptoms were mild and a half point was added if symptoms were severe; dead plants were rated 5 (Francis *et al.*, 2001).

For root dip inoculation, 15-day-old seedlings were pulled from the tray, secondary roots were trimmed to about 1 cm and dipped in a bacterial suspension (10^6 CFU/ml) for 15 min. Plants were transplanted in 50x20 cm rectangular pots (24 plants/pot) and maintained at the conditions specified above and observed for a 30 day period. For each strain, 24 plants were inoculated. Plants were scored for disease symptoms using a 1-5 disease scale developed for root inoculation by Poysa *et al.* (1993) with slight modifications: 0, no symptoms; 1, yellowing or slight wilting on leaves; 2, wilting on 1-2 leaves; 3, more than three leaves severely wilted; 4, all leaves severely wilted; 5, plant dead. The disease index (DI) was calculated using the following formula:

$$DI = \Sigma(\text{Severity rating} \times \text{Number of plants in that rating}) / \text{Total number of plants.}$$

The area under disease progress curve (AUDPC) was calculated as described by Campbell and Madden (1990). AUDPC and DI values were analyzed by one-way analysis of variance (ANOVA) with the CosTat 2.04 software (CoHort software, USA). Mean values were compared using Student-Newman-Keuls test ($P \leq 0.01$).

Reisolation of bacteria from tomato was performed on NGA. All inoculated material was autoclaved before discarding.

RESULTS AND DISCUSSION

Diagnosis of tomato bacterial canker. As mentioned, tomato plants from the greenhouses of the three farms surveyed showed TBC symptoms: unilateral wilting of the leaves and extensive discoloration of vascular tissues, but no typical stem cankers. Samples were analysed according to the EPPO protocol to detect the TBC agent *C. michiganensis* subsp. *michiganensis* using both PCR as presumptive diagnostic test and isolation on standard nutrient medium (OEPP/EPPO, 2005). Fifteen samples (five plants/farm) were repeatedly negative by PCR although the expected product of 614 bp was amplified from positive controls, i.e. stem portions of tomato plants artificially inoculated with *Cmm* (strain NCPPB382), tomato plant extracts additioned with a suspension of the same strain (spike samples) and DNA from strain NCPPB382.

In spite of the negative PCR results, typical *Cmm* colonies developed from all tomato samples on NGA within 3-4 days, appearing circular, pale yellow to yellow and semifluidal, thus undistinguishable from those

of the reference strain. Moreover, PCR amplification with CMM5/CMM6 from cell lysates of five colonies per plate randomly selected from seven different isolation plates was always negative.

When PCR was repeated from plant samples and controls using templates differentially prepared as detailed in Materials and Methods, none of the greenhouse samples gave positive results. Interestingly, a positive PCR response was only obtained from controls when the DNA was extracted using the DNA isolation kit and from ground plant stems, but not from those macerated in water as recommended in the EPPO protocol.

After purification by subculturing on NGA, thirty bacterial isolates (4, 13 and 13 from each farm, respectively) were further characterized. They were Gram-positive, and showed typical *Cmm* features: oxidative metabolism of glucose, catalase positive; oxidase negative; aesculin and starch hydrolysis positive, casein hydrolysis negative as *Cmm* strain NCPPB 382. All 30 isolates were negative by PCR when analysed with primers CMM5/CMM6, but were positive when tested with the other primer pair suggested by the EPPO protocol, PSA-4/PSA-R (Pastrik and Rainey, 1999), which amplify a 270 bp fragment of the 16S-23S rRNA intergenic spacer region, suggesting that the obtained isolates were probably *Cmm* strains.

Specific PCR amplification of atypical strains Two main virulence genes of *Cmm* are known in the reference strain NCPPB 382, *pat*-1 located on plasmid pCM2, encoding a serine protease, and *celA* on plasmid pCM1 coding for a cellulase. One explanation for the absence of the 614 bp fragment of the *pat*-1 gene amplified by CMM5/CMM6 from the *Cmm* strains isolated in the three farms could be provided by the loss of the *pat*-1 gene and potentially of the whole pCM2 plasmid. Although the presence of *pat*-1 negative strains has been previously reported, the virulence of these strains and their role in nature has not been investigated. The loss of this plasmid was reported only in the laboratory (Meletzus and Eichenlaub, 1991; Gartemann *et al.,* 2003), natural isolates mainly have different plasmid profiles (Kleitman *et al.,* 2008).

In order to further investigate the presence of *pat*-1 on pCM2 and to assess the presence of the other virulence gene *celA* on plasmid pCM1, a part of the *pat*-1-*rep* motif located downstream of *pat*-1 gene on pCM2 and the *celA* gene located on pCM1 were amplified by PCR using the primers P1rep/P3 rep (Dreier *et al.,* 1997) and PFC3/PFC5 (Kleitman *et al.,* 2008), respectively.

All tested (atypical and reference) *Cmm* strains showed the 552 bp *celA* amplification product. In atypical *Cmm* strains no amplification products were detected also with P1rep/P3rep, whereas PCR products ranging from 160 to 200 bp were amplified from the *Cmm* reference strains (Table 1). The absence of a P1rep/P3rep amplification product further suggests the lack of a pCM2-like plasmid in the *Cmm*-like strains. The different size of the amplicons from control strains was probably due to the variable number of direct repeats of the repetitive motif as previously reported by Dreier *et al.* (1997). These authors supposed that this sequence motif was involved in virulence gene expression suggesting that the repetitive sequence may protect the *pat*-1 mRNA from degradation by cellular endoribonuclease and 3'exoribonuclease (Dreier *et al.,* 1997).

The occurrence of strains lacking the *pat*-1 gene, and possibly lacking the entire pCM2-like plasmid carrying the gene, has been reported from Hawaii and Israel (Alvarez and Kaneshiro, 2005; Kleitmann *et al.*, 2008). In Israel, analyzing a large population of *Cmm* strains isolated from different locations from seeds and tomato plants during 1994 to 2007, it was found that about 20% of strains had lost the *pat*-1 gene, but had retained the *celA* gene (Kleitmann *et al.,* 2008). These strains were not virulent when inoculated on tomato (Kleitmann *et al.,* 2008). The absence of *pat*-1 was also found in several strains isolated from seeds that, according to their pathogenicity on tomato, were classified as: virulent, hypovirulent or avirulent (Alvarez and Kaneshiro, 2005; Alvarez *et al.,* 2005). The strains that showed reduced virulence (hypovirulent) only caused cankers in the plant, but did not induce wilting of the leaves.

pCM1 and pCM2 are conjugative plasmids (Gartemann *et al.*, 2003; Eichenlaub and Gartemann, 2011), and it was supposed that in a *Cmm* population in the xylem of tomato, the loss of the plasmid in by one cell could be compensated by the reacquisition of the plasmid from another cell carrying the plasmid. We suggest that the tomatoes of the three farms in this study were instead infected by a population of strains lacking pCM2 since *pat*-1 was not detected in any symptomatic plant and also the bacterial colonies recovered from these plants were PCR-negative.

Characterization of *Cmm*-like strains. The thirty isolates from the three farms were genotyped by BOX-PCR. They showed an identical genomic profile, which differed from that of reference strains, suggesting the presence of a clonal population in the area (Fig. 1). Three strains (one per farm) were identified as *C. michiganensis* subsp. *michiganensis* by the MicroLog3™ Microbial Identification system with similarity index values above 0.5. 16S rDNA fragments of about 1,000 bp were amplified and sequenced from two of the *Cmm*-like strains from two different locations (accession Nos: JQ917461, JQ917462). Based on BLASTn searches the sequences of the two *Cmm*-like strains showed 99-100% identity with the 16S rDNA sequences of other *Cmm* strains in database. Based on the results of presumptive diagnosis, the morphology on non-selective media, the identification by

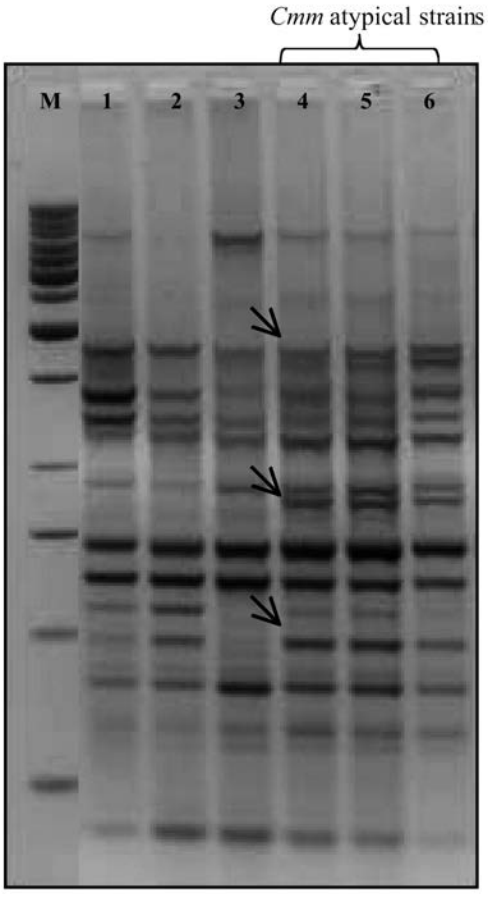

Fig. 1. Genomic fingerprinting of *Clavibacter michiganensis* subps. *michiganensis* strains obtained by BOX-PCR: lanes 1-3, reference strains *pat*-1 positive (PVCT156.1.1, PVCT163.2.1, PVCT171.1); lanes 4-6, representative of PCR negative *pat*-1 strains (PVCT162.2.1, PVCT160.2.1, PVCT161.6.1). M, GeneRuler 1 Kb ladder (Fermentas, Lithuania). Arrows show major difference of atypical strain population.

nutritional profile, and the PCR and 16S rDNA sequence analysis, we conclude that the tomatoes grown in the three greenhouses surveyed were infected by atypical *Cmm* strains probably lacking the pCM2 plasmid carrying the virulence gene *pat*-1 gene.

Pathogenicity tests. *Cmm* mutants lacking one or both plasmids lead to attenuated virulence phenotypes (Gartemann *et al.*, 2003). CMM100 is a plasmid-free derivative of strain NCPPB382, that was obtained by curing of the plasmids from wild type *Cmm* using high temperature (Meletzus and Eichenlaub, 1991). CMM100 can colonize tomato plants as efficiently as the parental strain NCPPB382, produce EPS, but is not able to induce disease on tomato. CMM101 and CMM102 lacking pCM2 and pCM1, respectively, show reduced virulence. Nevertheless contrasting results are available on the degree of virulence of *Cmm* field isolates lacking one of the two plasmids. Therefore, a pathogenicity trial with two inoculation methods was set up. A set of eight *Cmm* strains was selected: three *Cmm* atypical strains (one per location), three *Cmm* strains isolated from different greenhouses during 2005-2009, strain NCPPB382 and its plasmid-free derivative CMM100 (Table 1).

All strains except CMM100 were pathogenic regardless of the inoculation method. Mock-inoculated control plants did not show any symptoms. Although the two inoculation methods were suitable to assess the *Cmm* pathogenicity, the onset of typical symptoms was more rapid when plants were inoculated by leaf clipping rather than with root dip. The first symptoms appeared 8 dpi on the plants inoculated by leaf clip and only 16 dpi on those inoculated by root dip. Differences in virulence between the strains were observed with both inoculation methods, nevertheless leaf clip inoculation could be recommended for use in the official diagnostic protocols because of its faster response.

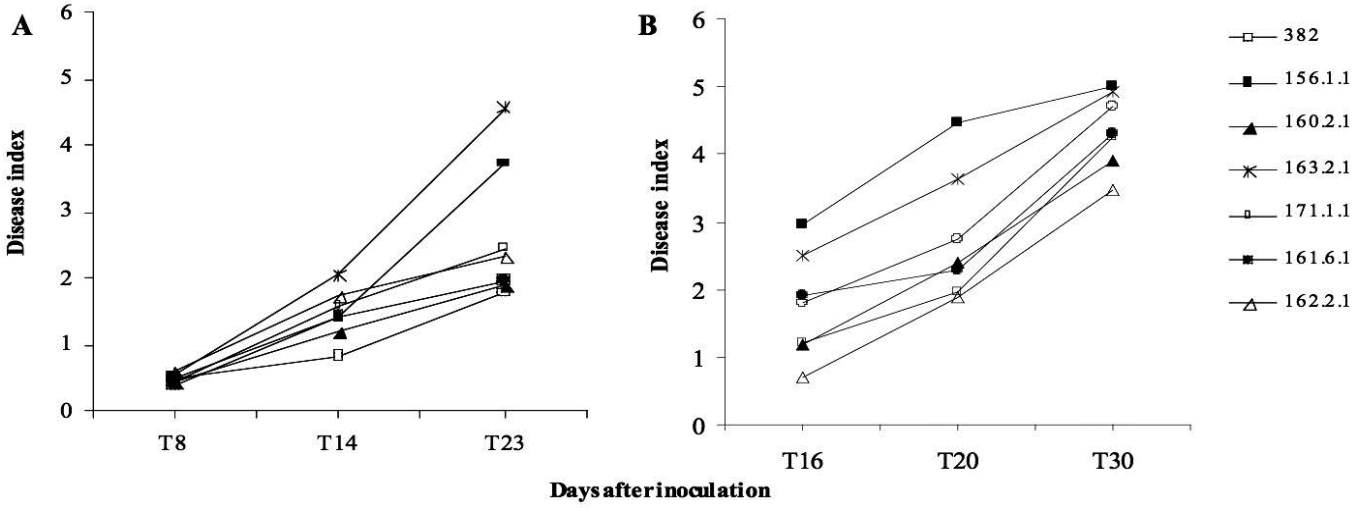

Fig. 2. Area under the disease progress curve (AUDPC) in tomato cv. Moneymaker inoculated by leaf clip (A) and root dip method (B).

In leaf clipping-inoculated plants, slight wilting of the leaves appeared already 8 dpi but typical symptoms (unilateral wilting of leaves and leaf necrosis, cankers on the stem) showed 14 dpi. *Cmm*-like strains induced symptoms indistinguishable from those of the reference strains. Moreover, DI and AUDPC values did not significantly differentiate the two groups of strains (with or without *pat*-1) (Table 2, Fig. 2A). However, at the last recording date (23 dpi) only 0-3% of the plants inoculated with *pat*-1-negative *Cmm* strains were dead (Table 2), whereas a death rate of 10-87% was registered in those inoculated with the other *Cmm* strains (NCPPB 382; PVCT 156.1.1; PVCT 163.2.1; PVCT 171.1.1) (Table 2). Thus, the severity of the disease is affected by the presence of *pat*-1. Nevertheless, *Cmm* isolates PVCT 163.2.1 and PVCT 156.1.1 were highly virulent displaying a significant increase in symptom severity compared to NCPPB 382 and PVCT 171.1.1.

Tomato plants inoculated by root-dip showed symptoms typical of vascular spread of the bacterium, i.e. unilateral wilting of one or more leaves or generalized wilting of the plant. None of the strains, not even the most virulent, elicited stem cankers. Although no significant differences were recorded in DI and AUDPC be-

Table 1. PCR characterization results of representative strains used in this study.

Strain number	Isolation date	Host/cultivar	PCR CMM5/6	P1rep/P3rep	PFC3/PFC5	PSA4/PSAR
PVCT 160.2.1	December 2006	Rovente	-	-	+	+
PVCT 161.6.1	January 2007	Rovente	-	-	+	+
PVCT 162.2.1	February 2007	Rovente	-	-	+	+
PVCT 171.1.1	February 2007	Italdor	+	+	+	+
PVCT 156.1.1	November 2006	n.d.	+	+	+	+
PVCT 163.2.1	March 2007	n.d.	+	+	+	+
NCPPB382	1956	n.d	+	+	+	+
CMM100			-	nt	-	+

Table 2. Disease development over time and area under the disease progress curve (AUDPC) values for tomato plants cv. Moneymaker after artificial inoculation by leaf clipping with seven *C. m.* subsp. *michiganensis* strains.

Strain number[1]	Disease index[2]						% of dead plants (T23)	AUDPC[2]	
	T8		T14		T23				
NCPPB382	0.50	A	0.84	A	1.79	A	10	16.47	A
PVCT160.2.1	0.47	A	1.24	AB	1.91	A	0	19.32	A
PVCT 161.6.1	0.38	A	1.43	AB	1.97	A	3	21.47	AB
PVCT 162.2.1	0.63	A	1.73	B	2.32	A	0	25.33	AB
PVCT 171.1.1	0,43	A	1.57	AB	2.64	A	27	24.93	AB
PVCT 156.1.1	0.52	A	1.41	AB	3.73	B	60	28.93	B
PVCT 163.2.1	0.58	A	2.07	B	4.57	C	87	37.80	C

[1] The strains in which the *pat*-1 gene was not detected are underlined. [2] Values in the columns with the same letter are n significantly different (P ≤0.01) by the Newman-Keuls test.

Table 3. Disease development over time and area under the disease progress curve (AUDPC) values in tomato plant cv. Moneymaker after artificial inoculation by the root dip method of seven *C. m.* subsp. *michiganensis* strains.

Strain number[1]	Disease index[2]						% of dead plants (T30)	AUDPC[2]	
	T16		T20		T30				
PVCT 162.2.1	0.71	A	1.88	A	3.46	A	20.8	31.83	A
PVCT 160.2.1	1.18	AB	2.39	A	3.91	AB	30.4	38.65	AB
PVCT171.1.1	1.21	AB	1.96	A	4.25	BC	54.1	37.38	AB
PVCT 161.6.1	1.92	BC	2.29	A	4.29	BC	54.1	41.33	B
NCPPB382	1.79	AB	2.75	A	4.71	CD	70.8	46.38	B
PVCT 163.2.1	2.50	CD	3.63	B	4.92	CD	62.5	54.96	C
PVCT 156.1.1	2.96	D	4.46	C	5.00	D	100	62.13	C

[1] The strains isolated in this study in which the *pat*- 1 gene was not detected were underlined. [2] Values in the columns with the same letter are not significantly different (P ≤0.01) by the Newman-Keuls test.

tween *Cmm*-like and reference strains, also with root-dip inoculation most of the plants (54-100%, depending on the strain) exposed to reference strains were dead 30 dpi, whereas only 21-54% of those inoculated with the atypical strains died (Table 3, Fig. 2B). Again, the strains PVCT 156.1.1. and PVCT 163.2.1 were significantly more virulent than all the other strains tested.

Strains lacking *pat*-1 were reported as non-pathogenic (Kleitman *et al.,* 2008) or placed in different virulence classes (Alvarez *et al.,* 2005). More typically, the *Cmm* strains collected in this study showed the same phenotype described for the mutant strain CMM101 possessing only pCM1. The mutant strain CMM101 caused disease on tomato but it took about 4 to 6 days longer for 50% of the plants to exhibit wilting symptoms (Meletzus and Eichenlaub, 1991; Meletzus *et al.*, 1993). Therefore, the strains in this outbreak are virulent and able to cause TBC both in the field and following artificial infections. Although they did not differ statistically from reference strains, wilting was delayed since with both inoculation methods more plants were still alive at the end of the plant assay.

The primary source of *Cmm* inoculum is represented by infected seeds. The *Cmm* strains without *pat*-1 investigated in this study were isolated from greenhouses in the same geographical area, from the same tomato cultivar and showed the same BOX-PCR genomic fingerprint. Moreover, they differed from some reference strains representative of a wider set of strains isolated in the same area in the same year (V. Catara, unpublished results). Phenotypic *Cmm* variants lacking both *pat*-1 and *celA* and the chromosomal *chpC, chpG, ppaA* or *tomA* genes, are highly virulent to pepper but still able to colonize and induce symptoms in tomato, opening to the question on the evolution of *Cmm* populations on particular hosts (Yim *et al.,* 2011). Since the presence in Sicilian *Cmm* strains of other virulence genes of the *chp/tom* region has been ascertained (V. Catara, unpublished results) studies are now underway for a better definition of the entire set of virulence genes and their variability in the strains in question.

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