RESEARCH ARTICLE

Molecular and phenotypic features of *Clavibacter michiganensis* subsp. *michiganensis* strains isolated from tomato in traditional cultivation

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Abstract

Tomato plants are attacked by various pathogenic bacteria, some of which can cause serious diseases in greenhouses and fields. During the summer of 2016–2018, several surveys were carried out after an outbreak of a disease in the tomato fields in northwest of Iran. To identify and characterize the disease's causal agent, sampling was done from symptomatic tomato plants that have dark brown to black lesions on the leaf margins, vascular discoloration and stem canker following wilting. Phenotypic features were checked out according to valid bacteriological texts. The pathogenic isolates were gram-positive, obligate aerobic, non-motile, and non-spore-forming with a variety in colony color of yellowish-orange. About 40% of pathogenic strains were virulent with a disease severity of more than 55%, other strains showed poor to moderated virulence. The results of the phylogenetic tree using partial sequences of 16 S rRNA gene and amplified fragments in PCR using three sets of specific primers confirmed the phenotypic results. The efficiency of PSA4/PSAR, PSA8/PSAR, and CMM5/CMM6 in detecting Cmm-like colonies strains was 59%, 84%, and 42%, respectively; however, CMM5/CMM6 could detect pathogenic Cmm strains. The results of the antibiogram test indicated that the studied strains were sensitive to 13 tested antibiotics, which this feature can be used in population diversity studies and providing disease management strategies. Based on the results of biochemical, physiological, and molecular experiments, the pathogenic strains isolated from tomatoes were *Clavibacter michiganensis* subsp. *michiganensis* (Cmm).

Introduction

Tomato (*Solanum lycopersicum* L., Family: Solanaceae) is one of the most consumed vegetable crops cultivated worldwide. Tomato is an excellent source of human nutrition and health, because of the richness of vitamins, minerals, and fibers (Kumar et al. 2022). The major tomato-growing countries are China, India, Turkey, The United States of America, Egypt, Italy, and Iran (FAOSTAT 2022). Tomato is cultivated in most regions of Iran, because of its favorable climate; especially in the northwest and south where it is produced traditionally. Several factors, such as insects, plant pathogens, weeds, climatic conditions, and unsuitable soils can reduce the yield of different tomato cultivars (Siddique et al. 2020). Up to now, 67 plant pathogenic agents including 15 bacteria that are known to cause diseases in tomato. Bacterial diseases are one of the main reasons for the decreasing quantity and quality of tomato yield in the world (Jones et al. 2014). *Clavibacter michiganensis* subsp. *michiganensis* (Cmm), *Xanthomonas vesicatoria, X. euvesicatoria, X. perforans, X. gardneri*, *Pseudomonas syringae* pv. *tomato* and *Ralstonia solanacearum* are the common tomato pathogens reported globally and can cause serious damages. Control of mentioned diseases is difficult and requires integrated disease management strategies to reduce the damage (Blancard 2012). Cmm is one of the most dangerous and economically important plant pathogens in the world, it is present the list of the quarantine plant pathogens of the European Union and many other countries in Asia, Africa, and the Caribbean (Blancard 2012; Sen et al. 2015).

For the first time in 1909, the disease was reported from a greenhouse in Michigan, US; and was described in 1910

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by Smith. As the disease is seed-borne, it spread rapidly to other states and epidemics occurred in the north and west of the US, and in some states of Canada in the decades of 1930, 1960, and 1980 (Borkar and Yumlembam 2017; Sen et al. 2015). Following the epidemics, research was conducted on various aspects of the disease. In Iran, according to existing reports tomato bacterial canker was reported from different parts of the country, and Cmm has a high potential for damage to this product (Nazari et al. 2007; Osdaghi et al. 2018).

Infected seed is the primary source of infection. In secondary dispersion, Cmm enters plant tissue through the stomata, hydathodes, roots, and damaged tissues, and it reaches the xylem vessels resulting in systemic infections (Valenzuela et al. 2018). Pathogenicity is mediated by virulence factors and transcriptional regulators encoded by the pathogenicity island (PAI) on a chromosome and two natural plasmids (pCM1 and pCM2) (Nandi et al. 2018). Cmm produces several types of degrading enzymes as virulence factors, such as serine proteases, cellulases, pectinases, and xylanases. These enzymes have essential roles in pathogenicity (Hwang et al. 2019). Cell wall hydrolyzing enzymes, especially cellulose, provide nutrient materials for the pathogenic bacterium (Bella et al. 2012; Valenzuela et al. 2021). Symptoms begin mainly as unilateral wilt of the leaves that later spread to entire the foliage. Cmm produces tiny, raised, and white blisters on the surface of young leaves and fruits. On the fruit's surface, the centers of the white blisters turn brown giving rise to "bird's eye" spots, and necrotic lesions at the edges of older leaves are usually visible. In a wilted plant, the color of the vascular tissues near the nodes changes to dark yellow or tan brown. Cankers develop on the stems and infected plants die in advanced stages (Bella et al. 2012; Siddique et al. 2020), although symptomless latent infections and the invasion of tomato seeds by Cmm are widespread (Nandi et al. 2018).

A yield reduction of up to 84% has been reported due to the tomato plants contaminated by Cmm. In vitro condition, the rate of disease damage varied from 46 to 93% (Sen et al. 2015). The prevalence of the disease symptoms in several greenhouses in Italy led to identify Cmm as the disease agent (Bella et al. 2012). Identification of Cmm was done by studying phenotypic traits and using primers PSA4/PSAR and PSA8/PSAR (Pastrik and Rainey 1999). In addition, the genetic diversity of the isolates was studied in rep-PCR using BOX, ERIC, and REP primers (Wassermann et al. 2017). Analysis of fatty acids via GC-FAME method and PCR using specific primers of CMM5/CMM6 were successfully used to identify Cmm isolates in the Mediterranean regions of Turkey (Basim and Basim 2018).

Preventing and management of seed-borne and soil-borne diseases such as tomato bacterial canker is very difficult. In addition, no available resistant cultivar has been introduced for this disease. Therefore, disease management should be done with a combination of preventive and control methods. Usage of certified seed, sterilizing equipment and crop rotation for at least 1–3 years, removing symptomatic seedlings and plants, deeply plowing infected derbies, spraying of copper-base compounds and antibiotics such as streptomycin are mentioned for the integrated management of the disease (Christopher Peritore-Galve et al. 2021; de Leon et al. 2011).

The purpose of this study was identifying the disease agent, which is the first and most crucial step in designing management strategies for the control or reducing the bacterial disease in tomato cultivation. For this, the identification of pathogenic isolates was performed based on phenotypic and molecular characteristics; and the phylogenetic relationship of the isolates was determined using 16 S rRNA gene sequence.

Materials and methods

Sampling and bacterial isolation

During the summer of 2016–2018, sampling of symptomatic tomato plants was done in infected fields in West Azarbaijan province, northwest of Iran. Disease symptoms include leaf spots and necrotic areas on leaves, discolored and brown lines in xylem vascular tissues, and stem canker. The symptomatic plants were placed in paper bags. During the transfer, the samples were placed in a chilled container, and were stored in a refrigerator at 4 °C. Bacteria were isolated from symptomatic leaves and stems. The infected leaves and stems were surface-disinfected by using 70% ethanol for 30 s and 2% sodium hypochlorite for 5 min and then washed thrice with sterile distilled water (SDW). Young spots on leaves were picked up with a disinfected scalpel blade, cut into 1–2 mm sections, and transferred to SDW. In the case of stems with canker symptoms, small sections of discolored vascular tissues were separated and transferred into SDW. After 15–20 min, serial dilutions were prepared and a loopful of the suspension 10^{-3} CFU mL^{-1} was cultured on nutrient broth yeast agar (NBYA) medium and Petri dishes were incubated at 28 °C for a week. A total of 387 bacterial isolations were done from infected samples in 48–96 h after incubation. Future experiments on isolates were carried out with thrice purified isolates by streak plate method on yeast dextrose carbonate agar (YDC) medium. Purified bacterial cultures were obtained with homogeneity colony morphology. For long-term storage, bacterial cultures were placed in nutrient broth with 25% glycerol and were stored at −80 °C. For routine experiments, bacterial isolates were cultured on nutrient agar (NA) medium or in Luria-Bertani broth (LB) medium at 28 °C for two days (Schaad et al. 2001).

Phenotypic experiments

Morphological, biochemical, and physiological features of studied bacteria were performed via standard bacteriological procedures. Tobacco (*Nicotiana tabacum* L.) and four o'clock flower (*Mirabilis jalapa* L.) were inoculated to record hypersensitivity reaction (HR) according to Ftayeh et al. (2010), Gram staining, colony morphology study on nutrient-broth yeast extract agar (NBY), and yeast dextrose carbonate agar (YDC) media were done. Further, the growth on triphenyl tetrazolium chloride agar (TTC) and on *Clavibacter nebraskense* selective (CNS) media was done. Levan, oxidase, motility, arginine dihydrolase, tyrosinase activity and hydrolysis of casein, esculin, gelatin, tween 80 and starch were carried out according to Schaad et al. (2001) and Klement et al. (1990). Aerobic/anaerobic growth, growth at 4 and 40 °C, production of H_2S from peptone, tolerance to 4, 5, and 7% NaCl (WV^{-1}), indole production, nitrate reduction, phosphatase, urease, lecithinase, and catalase were performed according to Schaad et al. (2001). The strain of *C. michiganensis* subsp. *michiganensis* NCPPB 382, provided by the National Collection of Plant Pathogenic Bacteria (UK), was used as a reference strain.

Pathogenicity and virulence tests

The infection procedure was performed following de Ftayeh et al. (2010). Tomato seedlings (cv. Super Stone) were grown in 20 cm diameter pots in a soil mix (containing sand, perlite, and peat compost in equal proportion) under natural daylight in the greenhouse. Six-week-old tomato seedlings were pierced on the main stem between the cotyledons and the first true leaf with a sterile scalpel that was dipped in a fresh bacterial colony. For virulence tests, 35 μL of the bacterial suspension in SDW of 10^9 CFU mL^{-1} was injected into the axil of the second or third true leaf. After inoculation, tomato plants were covered with clear polyethylene bags for 24 h at room temperature (18 °C). After removing the bags, infected plants were maintained in a greenhouse with a controlled climate at 28 °C under natural daylight with 80% relative humidity (RH). NCPPB 382 and SDW were used as positive and negative controls, respectively. Infected plants were monitored for vigor and developed symptoms up to 21 days after inoculation.

To fulfill Koch's postulates, the pathogen was re-isolated from the inoculated plants showing disease symptoms and re-identified via phenotypic tests including Gram staining, aerobic growth, colony morphology, and pigment production on YDC, as well as PCR using specific primers of CMM5/CMM6.

The strain's disease severity was assessed on the inoculated plants according to symptoms on a scoring of 1–5 (1—no disease symptom, 2—wilting in 1–25% of the plant, 3—wilting in 26–50% of the plant, 4—wilting in 51–75% of the plant, and 5—wilting in 76–100% of plant or plant death). Four replicates were considered for each strain (Klement et al. 1990). The experiment was repeated twice.

The disease severity (DS) for each strain was calculated using the following formula (Bella et al. 2012):

$$DS = \frac{\sum (n \times x_{1-5})}{N} \times 100,$$

where *DS* (%) is the percentage of disease severity, *n* is the number of plants in each numerical score, x_{1-5} is the numerical score, and *N* is the total number of evaluated plants for each strain.

Antibiogram

An antibiogram test was performed using sterile paper disks impregnated with antibiotics. Ten pathogenic Cmm strains from different regions and with different features in phenotypic, pathogenic, and virulence characters were selected for this test. The susceptibility reaction of selected strains was evaluated against 22 different antibiotics prepared from Padtan Teb Laboratory Instruments (Iran) (Table 1). 100 μL of bacterial suspension (1×10^9 CFU mL^{-1}) was spread over NA medium supplemented with 1% (WV^{-1}) glucose in 10 cm Petri dishes (Azucena et al. 2019). Then, antibiotic disks were placed 25 cm from each other on the medium and incubated at 28 °C for 48 h. A blank disk with SWD was used as the negative control. The inhibition zone was measured 48 h after incubation. The experiment was conducted with a completely randomized design (CRD). Four repeats were considered for each antibiotic disk. Statistical analysis

Table 1 Concentration of antibiotic in prepared disks used in antibiogram test

Antibiotic	Concentration (μg per disk)	Antibiotic	Concentration (μg per disk)
Amikacin	30	Imipenem	10
Amoxicillin	25	Meropenem	10
Ampicillin	10	Nalidixic acid	30
Cefalexin	30	Nitrofurantoin	300
Ceftizoxime	30	Novobiocin	30
Chloramphenicol	30	Penicillin	10
Cloxacillin	1	Rifampin	5
Doxycycline	30	Streptomycin	10
Erythromycin	15	Tetracycline	30
Furazolidone	100	Trimethoprim sulfamethoxazole	75.23
Gentamicin	10	Vancomycin	30

of data was performed by Tukey test, at the 99% level of confidence (p < 0.01), using SAS software (version 9.4). The experiment was repeated thrice.

Molecular identification

DNA extraction

Bacterial genomic DNA was extracted based on Li and de Boer ([1995](#)). A suspension of active bacterial colonies in 200 µL SDW was prepared. Then, 400 µL extraction buffer (Tris-HCl 50mM, pH: 8; EDTA 25mM; SDS 1%; Proteinase K 10 µg mL^{-1}) was added, and tubes were placed in Ban Mari at 55 °C for 3 h. Then, 400 µL ammonium acetate (7.5 M) was added to tubes, and DNA was recovered by isopropanol. DNA was re-dissolved in SDW after washing the plates with ethanol 70% twice. The quantity of DNA was adjusted to 50 ng µL^{-1}. The extracted DNA was stored at − 20 °C.

PCR using universal and specific primers

Molecular identification was done using a pair of universal primers fD1/rP2 and fD1/rD1 (Weisburg et al. [1991](#)) and three sets of specific primers including PSA4/PSAR (Pastrik and Rainey [1999](#)), PSA8/PSAR (PSA8 is a modification of PSA4), and CMM5/CMM6 (Dreier et al. [1995](#)) (Table [2](#)). FD1/rP2 and fD1/rD1 were derived from 16 S rRNA gene, PSA4/PSAR, and PSA4/PSAR were designed based on the 16-23 S rRNA intergenic spacer region, and CMM5/CMM6 was derived from a part of the *pat-1* gene localized on plasmid pCM2 (70.0 Kbp).

PCR reactions were performed with a 25 µL PCR cocktail containing 12 µL of Taq DNA Polymerase 2x Master Mix RED (Amplicon, Denmark), 1 µL of each primer (10 pmol µL^{-1}), and 1 µL of template DNA (50 ng). PCR amplification program for fD1/rP2 or fD1/rD1 primers was carried out under the following conditions; initial denaturation cycle at 94 °C (5 min), 35 cycles of denaturation at 94 °C (1 min), annealing at 62 °C (1 min) and extension at 72 °C (1.5 min), and a final extension at 72 °C for 7 min. PCR condition for specific primers was initial denaturation cycle at 94 °C (5 min), 30 cycles of denaturation at 94 °C (50 s), annealing at 58 °C (PSA4/PSAR and PSA4/PSA8) and 60 °C (CMM5/CMM6) (1 min) and extension at 72 °C (50 s), and a final extension at 72 °C for 7 min in a Palm cycler model GP001 (Corbett Research Co., Australia. For all samples, six µL of PCR product was applied in 1.0% agarose gel at 75 V for one hour, and Gene Ruler TM 1 kb DNA ladder was used as a marker. SDW was used as a negative control. Agarose gel 1.0% was stained by FluoroDye DNA Fluorescent Loading Dye 1 µL mL^{-1} (Smobio, Taiwan). Gels were photographed by a gel documentation system model ENDURO™ GDS (Industrial Labnet, USA).

Phylogenetic tree using 16 S rRNA sequence

Amplified DNA fragments were purified with an Expin™ Combo GP kit (GeneAll, South Korea), and sequenced using Sanger sequencing technology Macrogen Corporation (https://www.macrogenusa.com) (Seoul, South Korea). The sequences were edited using the Chromas software. To perform multiple sequence alignment, the GenBank database of nucleic acid sequences, which showed high similarity to extracted 16 S rRNA, was used. Alignment and comparison of the sequences were performed with ClustalX programs available on the bioinformatics web databases. The NCBI BLAST (the Basic Alignment Search Tool) database was used to analyze sequence homology for extracted 16 S rRNA gene sequences. Phylogenetic relations were inferred, by applying the Kimura-2-parameter model. The neighbor-joining (NJ) method and the adjacent method by MEGA 11 software (Tamura et al. [2021](#)) were used for the phylogenetic tree and tested by bootstrap (1000 repetitions).

Table 2 Traits of primers used in this study

Target	Primer	Sequence (5'–3')	Fragment size (bp)	References
ITS[a]	PSA4 (F)	TCATTGGTCAATTCTGTCTCCC	270	Pastrik and Rainey ([1999](#))
ITS	PSA8 (F)	TACTGAGATGTTTCACTTCCCC	270	ISF[b] method
ITS	PSAR (R)	TTGGTCAATTCTGTCTCCCTTC	270	Pastrik and Rainey ([1999](#))
pat-1	CMM5 (F)	GCGAATAAGCCCATATCAA	614	Burokiene ([2006](#))
pat-1	CMM6 (R)	CGTCAGGAGGTCGCCTAATA	614	Burokiene ([2006](#))
16 S rRNA	fD1 (F)	AGAGTTTGATCCTGGCTCAG	1500	Weisburg et al. ([1991](#))
16 S rRNA	rP2 (R)	ACGGCTACCTTGTTACGACTT	1500	Weisburg et al. ([1991](#))
16 S rRNA	rD1 (R)	AAGGAGGTGATCCAGCC	1500	Weisburg et al. ([1991](#))

[a] ITS, the region between 16 S rRNA and 23 S rRNA

[b] ISF method, available at: http://www.worldseed.org/cms/medias/file/TradeIssues/PhytosanitaryMatters/SeedHealthTesting/ISHI-Veg/TomatoCmm_010811.pdf

Results

Symptoms and phenotypic characteristics

Disease symptoms such as dark brown to black lesions on the edges of leaves, xylem discoloration and plant wilting, cankers on stems and petioles, and death of plants were observed in infected tomato plants (Fig. 1).

A total of 56 bacterial isolates out of 387 were found gram-positive, obligate aerobic, and non-motile. The colonies were semifluid and their color was yellow on YDC but different colors in a range of yellowish-orange colors were observed on the NBY medium. Hypersensitive reaction on tobacco and four o'clock flower leaves was positive with the formation of necrotic spots at the injection site of the isolates, but production of levan polymer, oxidase, arginine dihydrolase, nitrate reduction, and urease were negative. All isolates grew on TTC and CNS media, as well as NA containing NaCl 4–7%. Hydrolysis of starch, tween 80, and casein was negative but hydrolysis of aesculin was positive; and the reaction of isolates in gelatin liquefaction was variable. Other phenotypic results including carbon source utilization are listed in Table 3.

Pathogenicity and virulence

Results of the pathogenicity test on tomato seedlings indicated that 35 strains out of 56 Cmm strains were virulent with different degrees of disease symptoms on tomato seedlings. Other strains with positive HR on tobacco and four o'clock flower leaves did not show any disease symptoms on tomato plants. Among the virulent Cmm, the disease severity of 14 strains was more than 55%, so these strains were categorized as virulent strains. Disease severity from 21 to 55% was observed in 13 strains with moderated virulence. Other eight strains showed poor virulence with disease severity of less than 20% and in some cases, mild symptoms were noticed after two weeks (Table 4). Phenotypic features and PCR results of the re-isolated bacteria from inoculated plants proved Koch's postulates.

Molecular identification

To confirm the phenotypic identification, PCR was conducted using two sets of universal primers fD1/rP2 and fD1/rD1, and three sets of specific primers of PSA4/PSAR, PSA8/PSAR, along with CMM5/CMM6.

A 1500 bp fragment was amplified in all pathogenic and non-pathogenic Cmm strains (Fig. 2).

The retrieved results of 16 S rRNA sequences of the studied strains revealed 99.2–100% similarity to *C. michiganensis* subsp. *michiganensis* registered in GenBank (NCBI). Sequences of eight Cmm strains were deposited in GenBank databases under the accession numbers shown in Table 5.

A 270 bp fragment was amplified using PSA4/PSAR in 33 isolates out of 56 isolates which presented a typical Cmm colony; however, PSA8/PSAR primers identified 47 isolates by amplification of a fragment with a similar size (Fig. 3). On PCR using CMM5/CMM6 primers, 24 isolates were identified and the amplified fragment was 614 bp (Fig. 4) (Table 4).

A BLAST search in the GenBank database (NCBI) indicated that the sequences 16 S rRNA gene from the eight strains of this research had 99.2–100% homology with those of reference strains of *C. michiganensis* subsp. *michiganensis*. A Neighbor-joining phylogenetic tree of 16 S rRNA partial sequences from selected Cmm strains showed that the tomato isolates were grouped in two clads with 20 reference strains from the database. In clad A, the strains of cmm. Mi17, cmm.Ur42, cmm.Ur221, cmm.Ur147, cmm.Kh53, cmm.Ur9, and cmm.Mi82 were grouped with Cmm strains deposited from Germany, France, Italy, South Korea, Russia, China, Egypt, Japan, Kenya, Hungry, USA, and United Kingdom; in the clad B, cmm.Mh8 and four strains from South Korea and China were grouped. Both clads were separated from *Clavibacter michiganensis* pv. *nebrankensis* ATCC 27,822 which was used as an outgroup strain (Fig. 5).

Based on the results of phenotypic, pathogenic, and molecular experiments using universal and specific primers, and in comparison with bacterial characters described in valid bacteriological references, the pathogenic strain was

Fig. 1 Symptoms of bacterial canker disease on tomato plant. Necrosis on edge of leaves (**a**), plant wilting (**b**), stem canker and brown lines on xylem tissues of stem (**c**), leaves necrosis and stem canker (**d**), and dead infected plant by *Clavibacter michiganensis* pv. *michganensis* (**e**)

Table 3 Phenotypic features of pathogenic *C. michiganensis* subsp. *michiganensis* isolated from tomato plants

Characteristic	Reaction	Characteristic	Reaction
Gram reaction	Gram-positive	Phosphatase	+
Growth	Aerobic	Urease	–
Levan formation	–[a]	H₂S from peptone	+
Oxidase	–	Lecithinase	+
Motility	Non-motile	3-*Keto* lactose formation	–
Arginine dihydrolase	–	Utilization of:	–
HR (Tobacco and four o'clock flower)	+	L-Phenylalanine	–
Colony pigment on NBY[b] medium	Y/D[c]	D (+) Galactose	+
Colony pigment on YDC[d] medium	Y[e]	L-Arabinose	+
Growth on TTC[f] medium	+[g]	D-Arabinose	+
Growth on CNS[h] medium	+	Ribose	–
Gelatin hydrolysis	V[i]	Maltose	+
Aesculin hydrolysis	+	Lactose	+
Starch hydrolysis	–	Adonitol	–
Tween 80 hydrolysis	–	Fumarate	–
Casein hydrolysis	–	Inulin	–
Tyrosinase activity	–	D-Sorbitol	+
Growth at 4 °C	+	D-Xylose	+
Growth at 40 °C	+	L-Xylose	+
4% NaCl tolerance	+	Dulcitol	+
5% NaCl tolerance	+	L-Rhamnose	+
7% NaCl tolerance	+	Citrate	+
Catalase	+	Acetate	–
Nitrate reduction	–	Raffinose	+
Methyl red	–	D-Mannitol	+

[a]Negative reaction in more than 80% of strains

[b]Nutrient-broth yeast extract agar

[c]Yellow/diverse pigments within a range of yellow-orange colors

[d]Yeast dextrose carbonate agar

[e]Yellow

[f]2,3,5-Triphenyl tetrazolium chloride medium

[g]Positive reaction in more than 80% of strains

[h]*Clavibacter nebraskense* selective medium

[i]Variable reaction

subsequently identified as *Clavibacter michiganensis* subsp. *michiganensis*.

Antibiogram

In the antibiogram experiment, ten pathogenic Cmm strains showed different susceptibility to 22 studied antibiotics. Based on the results of variance analysis, a statistically significant difference at 1% level was observed among the studied antibiotics (Table 6).

Nalidixic acid, gentamicin, doxycycline, streptomycin, erythromycin, cloxacillin, rifampin, cephalexin, and furazolidone had the highest inhibition effect on bacterial growth compared to the control, respectively. Studied strains were resistant to other 13 antibiotics and no inhibition zone was formed around antibiotic discs (Fig. 6).

Discussion

Recently, typical symptoms of bacterial canker disease were observed in tomato fields in northwest of Iran. The pathogenic bacterial isolates obtained from symptomatic plants were relatively uniform and showed slight differences in phenotypic properties. Biochemical and physiological characteristics indicated that the strains belong to the subspecies of C. *michiganensis* subsp. *michiganensis*.

Several phenotypic characters such as colony pigment production on YDC, aesculin hydrolysis, and production

Table 4 Place and year of isolation, and severity of disease caused by pathogenic *Clavibacter michiganensis* pv. *michganensis* used in this study

Strain code	County of origin	Disease severity (%)	Degree of virulence[a]	Year of collection
cmm.Kh26	Khoi	45	2	2016
cmm.Kh32	Khoi	15	1	2016
cmm.Kh37	Khoi	38	2	2016
cmm.Kh53	Khoi	63	3	2018
cmm.Kh55	Khoi	100	3	2018
cmm.Mh3	Mahabad	12	1	2016
cmm.Mh8	Mahabad	72	3	2016
cmm.Mh13	Mahabad	26	2	2016
cmm.Mh24, cmm.Mh32	Mahabad	68	3	2016
cmm.Mi2	Miandoaab	83	3	2016
cmm.Mi17	Miandoaab	32	2	2016
cmm.Mi22	Miandoaab	20	1	2016
cmm.Mi28, cmm.Mi 35	Miandoaab	78	3	2017
cmm.Mi38	Miandoaab	55	2	2017
cmm.Mi62	Miandoaab	25	2	2017
cmm.Mi64	Miandoaab	82	3	2018
cmm.Mi82	Miandoaab	68	3	2018
cmm.Mi88	Miandoaab	26	2	2018
cmm.Ur9	Urmia	55	2	2016
cmm.Ur16, cmm.Ur58	Urmia	15	1	2016
cmm.Ur24, cmm.Ur52	Urmia	26	2	2016
cmm.Ur42	Urmia	100	3	2016
cmm.Ur93, cmm.Ur112, cmm.Ur119	Urmia	12	1	2017
cmm.Ur137	Urmia	54	2	2017
cmm.Ur152	Urmia	95	3	2017
cmm.Ur169	Urmia	25	2	2017
cmm.Ur221	Urmia	74	3	2018
cmm.Ur285	Urmia	30	2	2018
cmm.Ur302	Urmia	92	3	2018
Positive control (NCPPB382)	NCPPB[b]	67	3	
Negative control (SDW)		0		

[a]Degree of virulence: (1) poor virulence (disease severity less than 20%), (2) moderate virulence (disease severity from 21 to 55%), and (3) virulence (disease severity more than 55%)

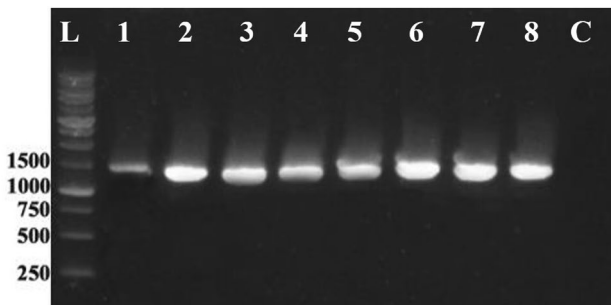[b] National Collection of Plant Pathogenic Bacteria (UK)

Fig. 2 The visualization of PCR amplification product from 16 S rRNA gene of *Clavibacter michiganensis* subsp. *michiganensis* strains. L) 1.0 Kb DNA ladder, (1) NCPPB382, (2) cmm.Ur.42, (3) cmm.Mh24, (4) cmm.Mi38, (5) cmm.Ur93, (6) cmm.Mh13, (7) cmm.Kh5, (8) cmm.Ur91, and C: negative control

Table 5 Accession numbers of 16 S rRNA sequences of selected *Clavibacter michiganensis* pv. *michganensis* in GenBank (NCBI)

Strain code	Accession number	Strain code	Accession numbers
cmm.Kh53	OM642114	cmm.Ur9	OM642129
cmm.Mh8	OM642117	cmm.Ur42	OM642132
cmm.Mi17	OM642116	cmm.Ur137	OM642131
cmm.Mi82	OM642121	cmm.Ur221	OM642133

of H_2S from peptone were similar to Cmm strains reported before; however, differences were observed in the results of gelatin liquefaction, starch hydrolysis, and phosphatase test (Nazari et al. 2007). The consumption of carbon
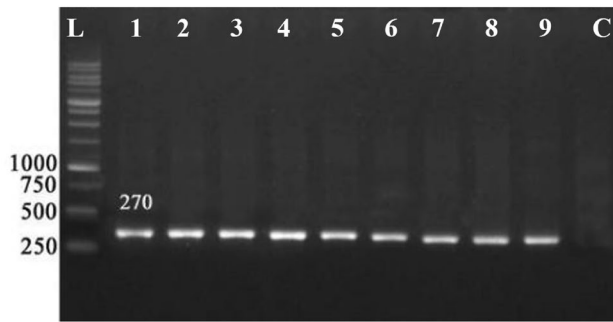
Fig. 3 Polymerase chain reaction analysis of ITS between 16 S rRNA and 23 S rRNA in pathogenic (1) NCPPB382, 2) cmm.Ur.16, (4) cmm.Mi88, 6) cmm.Kh55, (7) cmm.Mh13, (8) cmm.Mi.17) and non-pathogenic, (3) cmm.Mi80, (5) cmm.Ur.80, (9) cmm.Kh5) *Clavibacter michiganensis* subsp. *michiganensis* strains. L: 1.0 Kb DNA ladder, and C: negative control
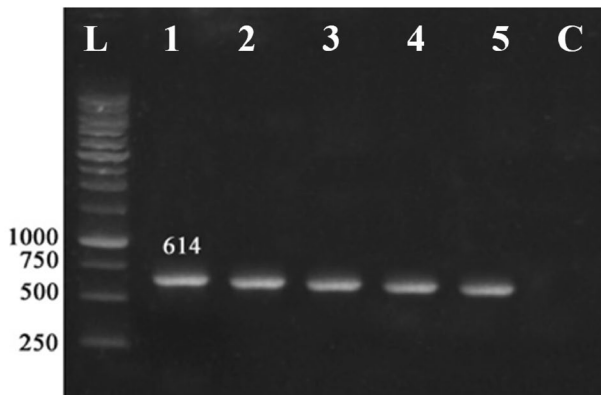

Fig. 4 Polymerase chain reaction analysis of *pat-1* gene in pathogenic *Clavibacter michiganensis* subsp. *michiganensis* strains. L) 1.0 Kb DNA ladder, (1) NCPPB382, (2) cmm.Kh53, (3) cmm.Mi17, (4) cmm.Ur9, (5) cmm.Mi88, and C) negative control

sources including lactose, maltose, mannitol, raffinose, dolisitol, and sorbitol by the strains was different from those of previous studies (Nazari et al. 2007). These results suggest that the differences in the physiological and biochemical characteristics among the strains can be due to the adaptation of pathogens to plant hosts; furthermore, various strains obtained from different regions indicate the genetic diversity of populations.

Chemical control of Cmm is limited to a few copper-based formulations and antibiotics. The frequent application of these materials faces the emergence of resistant strains. Resistance of plant pathogenic bacteria to antibiotics was reported in previous studies (de Leon et al. 2008; Sundin and Wang 2018; Valenzuela et al. 2019). Our results determine that there was a sensitivity of pathogenic Cmm strains to 22 antibiotics. The results indicated that

the strains are resistant to 13 antibiotics and these results can be useful in suggesting management strategies.

The pathogenic Cmm strains showed different degrees of disease severity on tomato seedlings. About 40% of pathogenic strains were determined as virulence strains, while other strains were moderate or poorly virulent. The pathogen-reducing effect can be due to the loss of genes involved in pathogenicity. More than ten genes such as *chpC*, *pat-1*, *phpA*, and *phpB* (encode serine-proteases), CAZymes (encode carbohydrate-active enzymes), *celA* and *celB* (encode cellulases), *xysA* and *xysB* (encode xylanases), *pelA1* and *pelA2* (encode pectinases), and *tomA* (encode tomatinase) are involved in the virulence of Cmm strains (Hwang et al. 2019; Nandi et al. 2018; Valenzuela et al. 2021).

Three sets of primer pairs were used for molecular identification of 56 gram-positive and HR-positive strains. Based on the target fragment amplification, the efficiency of PSA4/PSAR and PSA8/PSAR were 59% and 84%, respectively. PSA4/PSAR and PSA8/PSAR were designed according to a region between the 16–23 S rRNA intergenic spacer and 23 S rRNA gene, respectively; thus, these primers can identify pathogenic and non-pathogenic Cmm strains. The results of the current study are in agreement with those of Pastrik and Rainey (1999) that the modified primer pairs of PSA8/PSAR have more efficient in the detection of Cmm strains. CMM5/CMM6 was designed according to *pat-1* gene (Burokiene 2006) and allowed the identification of the pathogenic Cmm strains. This gene is localized on plasmid pCM2 and encodes an enzyme of 280 amino acids with a molecular mass of 29.7 kDa. Serine-protease is an essential enzyme for the virulence of Cmm strains (Dreier et al. 1995). In the present study, *pat-1* was detected in 42% of Cmm-like colonies strains, but this fragment did not amplify in 12 strains with positive pathogenicity test. According to the results of this study and previous findings (Bella et al. 2012), pathogenicity is controlled by several genes and related factors, as in some undetected strains with CMM5/CMM6, severe symptoms were observed in the pathogenicity test. Lastly, Valenzuela et al. (2021) revealed correlations between the degree of disease severity and the presence/absence of genes associated with virulence in pathogenic Cmm strains. Gene sequence analysis from the genomes of highly virulent strains indicated the presence of ten genes involved in virulence; while four of these genes, including *celB*, *xysA*, *pat-1*, and *phpA*, were not amplified in the strains causing mild symptoms. Yim et al. (2012) reported in PCR according to *pat-1* and *celA*, two plasmid-borne virulence genes, no fragment was amplified in Cmm strains isolated from the peppers. In addition, pathogenicity-related genes located on a pathogenicity island (PAI) including *chpC*, *chpG*, *ppaA*, or *tomA* genes were not detected in pathogenic Cmm strains isolated from pepper but these genes were detected in tomato strains.

Fig. 5 Neighbor-joining phylogenetic tree of 16 S rRNA partial sequences from selected *Clavibacter michiganensis* pv. *michiganensis* strains. Sequences of the compared strains were obtained from Gen-Bank. *Clavibacter michiganensis* pv. *nebrankensis* ATCC 27822 used as an out group strain. Bootstrap values was calculated from 1000 replicates
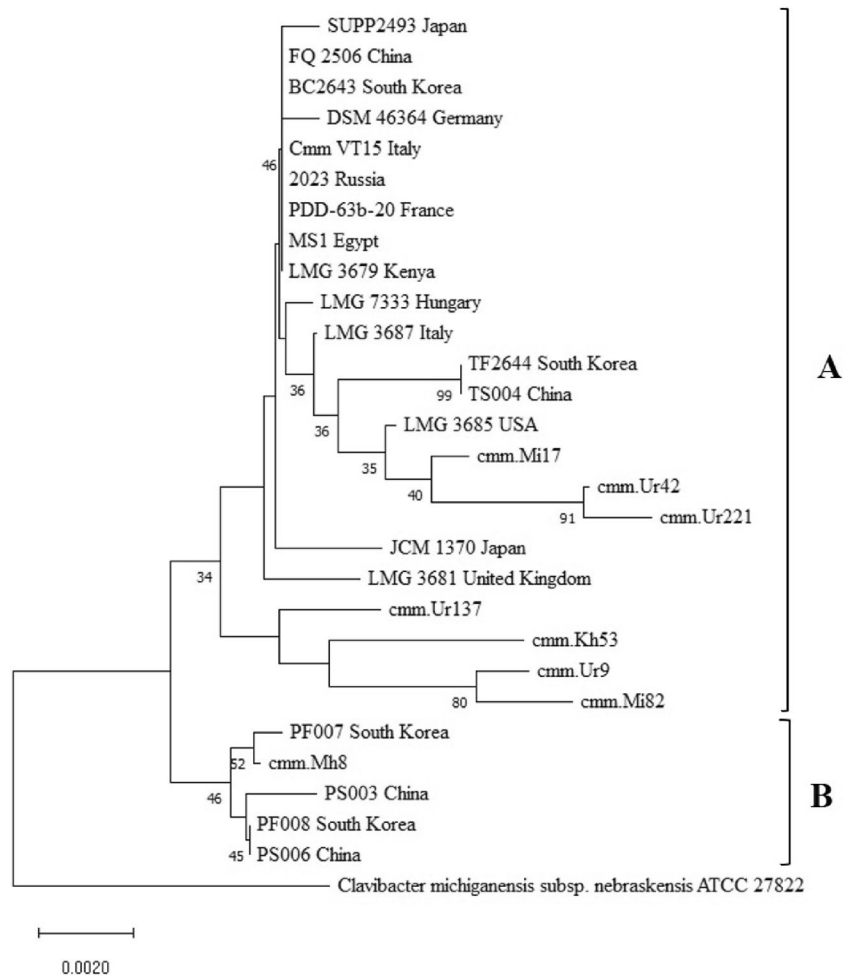
Table 6 Analysis of variance of antibiogram test

Source of variation	Degrees of freedom	Average of squares Diameter of inhibition zone
Antibiotic disk	22	155.27[**]
Error	46	0.942
Coefficient of variation (CV %)		21.60

[**]Statistically significant difference at 1% level

The phylogenic analysis based on 16 S rRNA gene of studied strains revealed high homology (99.2–100%) to *C. michiganensis* subsp. *michiganensis* registered in Gen-Bank (NCBI). Neighbor-joining phylogenetic tree of 16 S rRNA partial sequences indicated that the studied Cmm strains are similar to those strains from different countries including Germany, France, Italy, South Korea, Russia, China, Egypt, Japan, Kenia, Hungry, USA, and the United Kingdom. This finding emphasizes the importance of the disease transmission by infected seeds and its spread in different regions.

Based on the results of this study, the pathogenic agent of tomato disease in North West of Iran is *C. michiganensis* subsp. *michiganensis*. Considering the seed-borne nature of Cmm strains, quarantine restrictions should be applied to prevent its distribution to other tomato-producing areas of the country. In addition, the pathogenic strains showed resistance to some antibiotics. Information about the sensitivity characteristics of the pathogenic strains isolated in a geographical region against chemical compounds or antibiotics can benefit population diversity studies and provide low-risk strategies in plant disease management.

Fig. 6 Inhibition growth zone around antibiotic disks. Data are the average of ten *Clavibacter michiganensis* pv. *michganensis* strains in four replicates. Different letters represent significant differences at p < 0.01
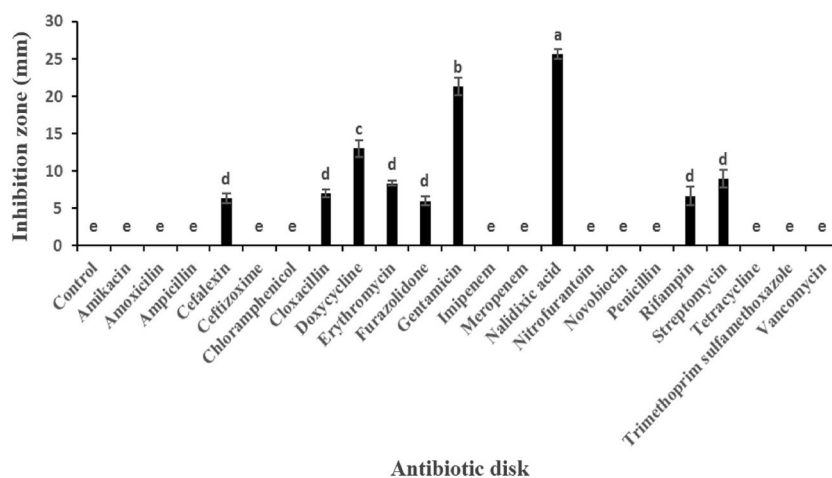
Author contributions The author Zahra Aghazadeh Soureh performed the experiments and corresponding author Maryam Khezri devised the experiment and drafted the manuscript.

Data availability All data generated or analyzed during this study are included in this published article.

Declarations

Conflict of interest The authors declare that they have no conflict of interest.

Ethical approval This article does not contain any studies with human participants or animals performed by any of the authors.

References

Azucena RCI, José Roberto CLJ, Martin ZR, Rafael CZ, Leonardo HH, Gabriela TP, Araceli CR (2019) Drug susceptibility testing and synergistic antibacterial activity of curcumin with antibiotics against enterotoxigenic *Escherichia coli*. Antibiotics 8(2):43. https://doi.org/10.3390/antibiotics8020043

Basim H, Basim E (2018) Phenotypic and genotypic characterization of *Clavibacter michiganensis* subsp. *michiganensis* causing tomato bacterial canker and wilt disease in Turkey. Eur J Plant Pathol 151:355–369. https://doi.org/10.1007/s10658-017-1378-3

Bella P, Ialacci G, Licciardello G, La Rosa R, Catara V (2012) Characterization of atypical *Clavibacter michiganensis* subsp. *michiganensis* populations in greenhouse tomatoes in Italy. J Plant Pathol 94(3):635–642. https://doi.org/10.4454/JPP.FA.2012.065

Blancard D (2012) Tomato diseases: identification, biology and control: a colour handbook, 2nd edn. CRC Press, London

Borkar S, Yumlembam R (2017) Bacterial diseases of crop plants. CRC Press, Boca Raton

Burokiene D (2006) Early detection of *Clavibacter michiganensis* subsp. *michiganensis* in tomato seedlings. Agron Res 4:151–154

Christopher Peritore-Galve F, Tancos MA, Smart CD (2021) Bacterial canker of tomato: revisiting a global and economically damaging seedborne pathogen. Plant Dis 105(6):1581–1595. https://doi.org/10.1094/PDIS-08-20-1732-FE

de Leon L, Siverio F, Lopez MM, Rodrıguez A (2008) Comparative efficiency of chemical compounds for activity against *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of tomato bacterial canker. Crop Prot 27(9):1277–1283. https://doi.org/10.1016/j.cropro.2008.04.004

de Leon L, Siverio F, Lopez MM, Rodriguez A (2011) *Clavibacter michiganensis* subsp. *michiganensis*, a seedborne tomato pathogen: healthy seeds are still the goal. Plant Dis 95(11):1328–1338. https://doi.org/10.1094/PDIS-02-11-0091

Dreier J, Bermpohl A, Eichenlaub R (1995) Southern hybridization and PCR for specific detection of phytopathogenic *Clavibacter michiganensis* subsp. *michiganensis*. Phytopathology 85(4):462–468. https://doi.org/10.1094/Phyto-85-462

FAOSTAT Statistics Database (2022) The agricultural production domain. http://www.fao.org/faostat/en/#data/QCL. Accessed 22 May 2022

Ftayeh RM, von Tiedemann A, Koopmann B, Abu-Ghorrah M, Rudolph K (2010) Occurrence of *Clavibacter michiganensis* subsp. *michiganensis*, the causal agent of bacterial canker of tomato in Syria. Phytopathol Mediterr 49(2):172–178. https://doi.org/10.14601/Phytopathol_Mediterr-8364

Hwang IS, Oh EJ, Lee HB, Oh CS (2019) Functional characterization of two cellulase genes in the Gram-positive pathogenic bacterium *Clavibacter michiganensis* for wilting in tomato. Mol Plant Microbe Interact 32(4):491–501. https://doi.org/10.1094/MPMI-08-18-0227-R

Jones JB, Zitter TA, Momol TM, Miller SA (2014) Compendium of tomato diseases. APS Press, Manhattan

Klement Z, Rudolph K, Sands DC (1990) Methods in phytobacteriology. Akademiai Kiado, Budapest

Kumar M, Chandran D, Tomar M, Bhuyan DJ, Grasso S, Sa AGA, Carciofi BAM, Radha, Dhumal S, Singh S, Senapathy M, Changan S, Dey A, Pandiselvam R, Mahato DK, Amarowicz R, Rajalingam S, Vishvanathan M, Saleena LAK, Mekhemar M (2022) Valorization potential of tomato (*Solanum lycopersicum* L.) seed: nutraceutical quality, food properties, safety aspects, and application as a health-promoting ingredient in foods. Horticulturae 8(3):265. https://doi.org/10.3390/horticulturae8030265

Li X, de Boer SH (1995) Selection of primers for polymerase chain reaction primers from an RNA intergenic spacer region for specific detection *Clavibacter michiganensis* subsp. *Sepidonicus*. Phytopathology 85(8):837–842. https://doi.org/10.1385/0-89603-244-2:31

Nandi M, Macdonald J, Liu P, Weselowski B, Yuan ZC (2018) *Clavibacter michiganensis* ssp. *michiganensis*: bacterial canker of tomato, molecular interactions and Disease management. Mol Plant Pathol 19(8):2036–2050. https://doi.org/10.1111/mpp.12678

Nazari F, Niknam GR, Ghasemi A, Taghavi SM, Momeni H, Torabi S (2007) An investigation on strains of *Clavibacter michiganensis* subsp. *michiganensis* in north and north west of Iran. J Phytopathol 155(9):563–569

Osdaghi E, Ansari M, Taghavi SM, Zarei S, Koebnik R, Lamichhane JR (2018) Pathogenicity and phylogenetic analysis of *Clavibacter michiganensis* strains associated with tomato plants in Iran. Plant Pathol 67(4):957–970. https://doi.org/10.1111/ppa.12801

Pastrik KH, Rainey FA (1999) Identification and differentiation of *Clavibacter michiganensis* subspecies by polymerase chain reaction-based techniques. J Phytopathol 147(11–12):687–693. https://doi.org/10.1046/j.1439-0434.1999.00442.x

Schaad NW, Jones JB, Chun W (2001) Laboratory guide for identification of plant pathogenic bacteria, 3rd edn. APS Press, Manhattan

Sen Y, van der Wolf J, Visser RG, van Heusden S (2015) Bacterial canker of tomato: current knowledge of detection, resistance, and interactions. Plant Dis 99(1):4–13. https://doi.org/10.1094/PDIS-05-14-0499-FE

Siddique M, Din N, Ahmad M, Ali A, Naz I, Alam SS, Ullah N (2020) Bio efficacy of some aqueous phytoextracts against *Clavibacter michiganensis* subsp. *michiganensis* (smith), the cause of bacterial canker of tomato. Gesunde Pflanzen 72:207–217. https://doi.org/10.1007/s10343-020-00503-9

Sundin GW, Wang N (2018) Antibiotic resistance in plant-pathogenic bacteria. Annu Rev Phytopathol 56:161–180. https://doi.org/10.1146/annurev-phyto-080417045946

Tamura K, Stecher G, Kumar S (2021) MEGA11: molecular evolutionary genetics analysis version 11. Mol Biol Evol 38(7):3022–3027. https://doi.org/10.1093/molbev/msab120

Valenzuela M, Besoain X, Durand K, Cesbron S, Fuentes S, Claverias F, Jacques MA, Seeger M (2018) *Clavibacter michiganensis* subsp. *michiganensis* strains from central Chile exhibit low genetic diversity and sequence types match strains in other parts of the world. Plant Pathol 67(9):1944–1954. https://doi.org/10.1111/ppa.12911

Valenzuela M, Mendez V, Montenegro I, Besoain X, Seeger M (2019) Streptomycin resistance in *Clavibacter michiganensis* subsp. *michiganensis* strains from Chile is related to an rpsL gene mutation. Plant Pathol 68(3):426–433. https://doi.org/10.1111/ppa.12971

Valenzuela M, Gonzalez M, Velasquez A, Dorta F, Montenegro I, Besoain X, Salva-Serra F, Jaen-Luchoro D, Moore ERB, Seeger M (2021) Analyses of virulence genes of *Clavibacter michiganensis* subsp. *michiganensis* strains reveal heterogeneity and deletions that correlate with pathogenicity. Microorganisms 9(7):1530. https://doi.org/10.3390/microorganisms9071530

Wassermann E, Montecchia MS, Correa OS, Damian V, Romero AM (2017) *Cbacter michiganensis* subsp. michiganensis strains virulence and genetic diversity. A first study in Argentina. Eur J Plant Pathol 149:35–42. https://doi.org/10.1007/s1065-017-1159-z

Weisburg WG, Barns SM, Pelletier DA, Lane DJ (1991) 16S ribosomal DNA amplification for phylogenetic study. J Bacteriol 173(2):697–703. https://doi.org/10.1128/jb.173.2.697-703.1991

Yim KO, Lee HI, Kim JH, Lee SD, Cho JH, Cha JS (2012) Characterization of phenotypic variants of *Clavibacter michiganensis* subsp. *michiganensis* isolated from *Capsicum annuum*. Eur J Plant Pathol 133:559–575. https://doi.org/10.1007/s10658-011-9927-7