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Clavibacter michiganensis subsp. *michiganesis*: Tracking strains using their genetic differentiations by ISSR markers in Southern Turkey

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ABSTRACT

There is no available data published related to the dissemination of bacterial canker caused by *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) and its genetic diversity in Turkey. It is important to control new introduced inoculum sources by commercial seeds and seedlings. Pathogens were identified by morphological features and the identity was confirmed by PCR amplification using a specific primer PSA-4, PSA-R in addition to microbiological tests. ISSR markers showing high polymorphism ratios were selected and used to characterize Cmm strains. The collected strains were classified into different groups on the basis of ISSR-PCR fingerprints, which showed remarkable genetic specificity and diversity not previously identified in Cmm, suggesting that genetic differences are related to dissemination of the pathogen in the region. This is the first ever study carried out on the characterization of Cmm using ISSR. The selected ISSR primers to characterize Cmm can be used to determine genetic differences in further studies.

1. Introduction

Commercial production of tomato in Turkey is intensively practiced in protected fields and local greenhouses. The Gram-positive bacterium *Clavibacter michiganensis* subsp. *michiganensis* (Cmm) is the causal agent of canker and wilt disease in tomato (*Solanum lycopersicum)* [9]. It causes severe losses and devastation around the world in many tomato-growing areas. The bacterial pathogen was originally described in 1910 as the cause of bacterial canker of tomato in North America. The pathogen is now present in all main production areas of tomato and is quite widely distributed in the EPPO region [10]. Cmm penetrates a plant through wounds or natural openings, reaches the xylem, and develops a massive systemic infection [19]. Moreover, symptomless greenhouse transplants may serve as a reservoir of Cmm populations. These are the latent infections resulting in plant wilting, stunting, reduced yield, and eventually plant death. Use of certified seeds and healthy transplants is recommended for preventive cultural management. Disinfection of the greenhouse, removal of plant debris or plowdown and rotation with non-Solanaceous plants for at least two years has been suggested as a good management strategy [14]. Resistant tomato cultivars also seem to be the most efficient control measure against Cmm. Sources of resistance to the bacterial canker pathogen have been found in several wild relatives of *Lycopersicon esculentum*: *Lycopersicon peruvianum*, *Lycopersicum hirsutum* and *Lycopersicon pimpinellifolium* [20]. Growers have limited options in managing bacterial canker in the field using chemical control [19]. Some growers apply copper compounds, either alone or mixed with mancozeb, preventively or after symptoms develop on plant foliage [18]. The application of copper compounds or their mixtures with the protective fungicide mancozeb in the field has been recommended for five or seven day intervals to reduce the severity of canker symptoms and fruit spotting [32]. The application of copper-based bactericides on tomato seedlings in the greenhouse reduces the population size and spread of Cmm and impacts plant development and yield in the field [18,38]. However, frequent use of these bactericides may lead to the occurrence of copper-resistant bacterial strains [8]. Disease control is feasible when antibiotics such as streptomycin are applied, although their use is forbidden in most countries [6]. Cmm is a quarantine organism under the European Union Plant Health legislation [2] and is also in the pest quarantine

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list of the Plant Protection Inspection Service in Turkey. Infected seed is the main inoculum source leading to outbreaks and dissemination of Cmm infection [34]. The population levels of the pathogen in/on the seed becomes very low [17]. Therefore, in addition to conventional detection methods, PCR-based detection methods have been developed [28,30]. Since the pathogen is seed-borne [32] a serious epidemic resulting from the transmission from seed to seedlings in tomato-growing fields is possible [13]. Therefore, genetic pathogen mapping is an important step in disease control and to follow the flow of new inoculum sources in a country. On the other hand, broad morphological variation among Cmm strains and the variable level of seed infection have increased the difficulty of developing a consistent and reliable detection method with uniform sensitivity and reproducibility. In a recent study, molecular typing and spread of Cmm in greenhouses in Japan was reported as an epidemiological study by rep-PCR using spatial pattern by Morisita-index, suggesting that mainly infection resulting from soil-borne and disbudding and defoliation in Japan [21].

ISSR-PCR fingerprint is also another helpful informative tool in epidemiological studies. To know current genetic profiles of the pathogen within isolates is important to track new and different sources of transmission. ISSR (Inter Simple Sequence Repeat) is a general term for a genome region between microsatellite loci. ISSR-PCR is a fast and inexpensive genotyping technique with a wide range of uses, including the characterization of genetic relatedness among populations [1,12,16,24,36,37]. The complementary sequences to two neighboring microsatellites are used as PCR primers; the variable region between them becomes amplified. The limited length of amplification cycles during PCR prevents excessive replication of overly long contiguous DNA sequences, so the result will be a mix of a variety of amplified DNA strands which are generally short but vary considerably in length [15,24–27,33].

To the best of our knowledge this the first study in which genetic diversity of plant pathogenic bacteria can be evaluated by ISSR markers. The aims of this study were to evaluate genetic differences within collected Cmm isolates in our region and to develop a fingerprint method to follow the source of contamination to detect the occurrence of an epidemic. Moreover, our findings efficiently demonstrate the use of ISSR markers, which are as reproducible as AFLP in addition to being more cost effective. This is the first ever study to characterize Cmm strains using ISSR.

2. Material and methods

2.1. Bacterial strains and culture media

The Cmm strains used in this study are listed in Table 1. The strains were identified according to [11,31]. The identified strains were obtained both from the collection of the Çukurova University Plant Protection Department and BATEM Institute, Antalya. The geographical origins of the isolates were given in Fig. 1. The bacteria were maintained on plates containing nutrient agar (NA; Merck, Germany) and incubated at 28 °C for 48–72 h. Strains were stored for short periods at 4 °C and for long periods at −80 °C in 40% glycerol.

2.2. Pathogen confirmation by specific PCR

100 μL bacterial suspension was spread onto NBY medium (used contained per liter of distilled water; Difco Nutrient broth 8 g; Difco yeast extract 2 g; glucose 10 g; K_2HPO_4 2 g; KH_2PO_4 0.5 g; $MgSO_4.7H_2O$ 0.2 g; agar 20 g. and pH adjusted to 7.0) and incubated on an orbital shaker (80 rpm) at 28 °C for 48 h. The culture was harvested by adding 1 mL of water and suspending the cells with a glass-loop. The PCR protocol suggested in EPPO standards PM 7/42 of [28] was used to confirm the pathogen by PCR using 25 μL amplicons. For PCR, from each presumptive isolate and from a culture of the reference strain, a single colony was suspended in 100 μL of sterile distilled water in vials. Closed vials were heated at 95 °C for 15 min. Heated suspensions were transferred onto ice for cooling. A 10 μL aliquot of the bacterial suspension was added to the PCR reaction as template DNA. Amplifications were performed in a 96-well GeneAmp® PCR System 9700 (Applied Biosystems) using forward primer PSA-4 (5′-TCA TTG GTC AAT TCT GTCTCC C-3′) and reverse primer PSA-R (5′-TAC TGA GAT GTT TCA CTT CCC C-3′) [2]. Denaturation was done at 94 °C for 5 min, followed by 40 cycles of 1 min at 94 °C, 1 min at 62 °C for and 30 s at 72 °C; and a final extension for 5 min at 72 °C. A Cmm reference strain (ICPM, 7200) was used as a positive control obtained from PCR and water served as the negative control. For direct PCR and from extractions, the negative control consisted of samples without the addition of infected material. The amplified products were subjected to electrophoresis in a 1.5% agarose gel and stained with ethidium

Table 1
List of 21 samples of *Clavibacter michiganensis* subsp. *michiganensis* isolated from (a) Antalya, (b) Kumluca, (c) Mersin and (d) Manavgat.

	Samples	Collection site geographic distance of site	The field locations	Tomato varities for authmn/spring season	Isolated from seed, seedlings/grafted seedling	Year
1	A	Antalya (a)	Çalkaya	Authmn	Seedling	2008
2	B	Antalya (a)	Kocayatak	Authmn	Seed	2007
3	C	Antalya (a)	Aşağıkocayatak	Authmn	Seed	2008
4	D	Antalya (a)	Kumköy	Spring	Seedling	2008
5	E	Antalya (a)	Solak	Spring	Seedling	2009
6	F	Antalya (a)	Dumanlar	Authmn	Seedling	2007
7	G	Antalya (a)	Kocayatak	Authmn	Seedling	2008
8	H	Antalya (a)	Serik	Authumn	Seed	2008
9	I	Antalya (a)	Alaylı	Spring	Grafted seedling	2008
10	J	Kumluca (b)	Hasyurt	Spring	Seedling	2008
11	K	Kumluca (b)	Mavikent	Spring	Seedling	2008
12	L	Kumluca (b)	Hacıveliler	Authmn	Grafted seedling	2008
13	M	Kumluca (b)	Beşikci	Spring	Seedling	2009
14	N	Kumluca (b)	Beykonak	Spring	Seedling	2008
15	O	Kumluca (b)	Sahilkent	Authmn	Seedling	2008
16	P	Kumluca (b)	Beykonak	Authmn	Grafted seedling	2009
17	Q	Mersin (c)	Şahmurdu	Authmn	Seedling	2005
18	R	Mersin (c)	Canboğazlı	Authmn	Seedling	2005
19	S	Mersin (c)	Tapureli	Authmn	Seedling	2005
20	T	Mersin (c)	Tarsus	Authmn	Seedling	2005
21	U	Manavgat (d)	Dikmen	Spring	Seedling	2008