Journal of Agricultural Science and Technology (2025) Vol. 27 (4): 791-803

Characterization of Causal Agents of Bacterial Spot on Tomato Fields in Iğdır Plain (Turkey)

Mustafa Akbaba[1*], Mesude Figen Donmez[2], and Kaan Hurkan[3, 4]

ABSTRACT

Bacterial spot of tomatoes, a serious disease, significantly reduces tomato yields in Turkey and many other countries. In Iğdır Plain, this study was aimed to characterize bacteria that are causal agents of spot disease in tomatoes. Symptomatic tomato plants were collected from fields within the plain for bacterial isolation. Ten bacterial strains belonging to the genus *Xanthomonas* were isolated from different parts (leaves and fruits) of tomato plants. Through a series of analyses encompassing pathogenicity assessments, biochemical assays, FAME profiling, PCR using species-specific primers, and phylogenetic analysis of *HrpB* gene sequences, the strains were conclusively identified as *Xanthomonas euvesicatoria* pv. *euvesicatoria* and *X. euvesicatoria* pv. *perforans*. According to our results, FAME were not effective in distinguishing these two species (*X. euvesicatoria* pv. *euvesicatoria* and *X. euvesicatoria* pv. *perforans*). In addition, only the primer BS-XeF/BS-XeR detected *X. euvesicatoria* pv. *euvesicatoria* and other primers, i.e. Bs-XpF/Bs-XpR were not able to detect some bacterial strains. To our knowledge, while bacterial spot disease in previous studies was reported in Iğdır Plain, this study marks the pioneering identification of *X. euvesicatoria* pv. *euvesicatoria* and *X. euvesicatoria* pv. *perforans* as the precise causative agents of the disease in tomatoes cultivated in this area. Additionally, *X. euvesicatoria* pv. *perforans* strain XCV2 was identified as the most virulent strain in this study. It caused a severity rate of 74% in tomato (cv. 'Süper domates').

Keywords: Bacterial spot, FAME, *HrpB* gene, Tomato, *Xanthomonas*.

INTRODUCTION

Tomato (*Solanum lycopersicum* L., formerly, *Lycopersicon esculentum* Mill.) is one of the most commercially important vegetables cultivated worldwide (Padmanabhan *et al.*, 2016). Tomato production is estimated to be approximately 186 million tons in 2022. Türkey, with an annual production of approximately 13 million tons, ranks third in global tomato production, after China and India (FAO, 2022).

Iğdır Plain is a low-lying area (between 800-900 meters above sea level) in eastern Turkey, bordering Armenia, Azerbaijan and Iran (Aydın and Çelik, 2019). The surrounding mountains create a unique microclimate with favourable temperatures for agriculture (Yaltı and Aksu, 2019). This allows for a diverse range of crops to be grown there. Tomatoes are the leading vegetable crop on the Iğdır Plain, boasting an annual yield of 35,217 tons (Türkiye İstatistik Kurumu, 2022).

Xanthomonas is a genus of Gram-negative bacteria that includes several important plant

[1] Department of Plant Protection, Faculty of Agricultural Sciences and Technology, Sivas University of Science and Technology, 58140, Sivas, Turkiye.
[2] Department of Plant Protection, Faculty of Agriculture, Igdir University, 76000, Igdir, Turkiye.
[3] Department of Agricultural Biotechnology, Faculty of Agriculture, Igdir University, 76000, Igdir, Turkiye.
[4] Research Laboratory Practice and Research Center, Igdir University, 76000, Igdir, Turkiye.
*Corresponding author; e-mail: mustafa.akbaba@outlook.com

pathogens. These bacteria are obligate aerobes that obtain their energy through chemoorganotrophy (Leyns *et al.*, 1984). A significant proportion of *Xanthomonas* species exhibit plant pathogenic properties, causing infections in a wide range of monocotyledonous and dicotyledonous crops (Büttner and Bonas, 2010). These include economically important food crops such as tomato and pepper (Kebede *et al.*, 2014; Potnis *et al.*, 2015). Tomato bacterial spot is caused by a complex of *Xanthomonas* species, primarily *X. euvesicatoria*, *X. vesicatoria*, *X. perforans*, and *X. gardneri* (Jones *et al.*, 2004). Recently, *X. euvesicatoria* and *X. perforans* are now re-clasified two pathovars of the same species, *X. euvesicatoria* pv. *euvesicatoria (*formerly *X. euvesicatoria)* and *X. euvesicatoria* pv. *perforans (*formerly *X. perforans)*, respectively (Constantin *et al.*, 2016). Meanwhile, *X. gardneri* has been reclassified as *X. hortorum* pv. *gardneri* (Morinière *et al.*, 2020). Only *X. vesicatoria* remains the same position (Osdaghi *et al.*, 2021). These four *Xanthomonas* species cause significant economic losses on tomato production in both field and greenhouse settings and remain the major limiting factors for tomato production (EPPO, 2013). Tomato bacterial spot disease causal agents exhibit a global distribution, affecting all aerial parts of the plant (Potnis *et al.*, 2015). Infected seeds, volunteer crop plants, and diseased plant debris are the primary sources of inoculum for tomato bacterial spot. The bacteria can be dispersed by rain and/or sprinkler irrigation droplets driven by the wind within fields and from nearby areas (Jones *et al.*, 1991). Symptoms of disease appear on various plant organs, including leaves, fruits, and stems (Jones *et al.*, 2013). Disease-caused yield reduction is a consequence of a diminished photosynthetic leaf area, dropped buds and flowers, and decreased commercial fruit quality. Severe infections can cause direct fruit yield losses of 23% to 44%. Additionally, infected plants shed leaves, exposing fruits to damaging sunlight and causing sunscald, leading to further indirect losses (Bashan *et al.*, 1985).

All the four species are on a "high risk" list maintained by the European and Mediterranean Plant Protection Organization (EPPO) with unique codes: XANTEU, XANTGA, XANTPF, and XANTVE. They are classified as EPPO category A2 (no. 157) and are considered a significant threat within the European Union (EU Annex II/A2). Hence, they are under strict quarantine control and zero tolerance all over the world (EFSA Panel on Plant Health, 2014; EPPO, 2013). Bacterial spot disease has been reported in many pepper and tomato producing areas in Türkey (Kayaaslan *et al.*, 2023).

Researchers have used species-specific primers to identify *Xanthomonas* species causing bacterial spot (Koenraadt *et al.*, 2009). Also, analysis of the partial *hrpB* gene sequence was considered a valuable tool for differentiating between *Xanthomonas* species at the species level (Obradovic *et al.*, 2004; Young *et al.*, 2008). By combining these two methods – species-specific primers and partial *hrpB* gene sequencing – researchers can achieve highly accurate identification of the four *Xanthomonas* species known to cause bacterial spot disease in tomatoes.

No previous study has characterized the bacterial species on tomato in Iğdır Plain. Thus, this study focused on characterizing bacterial spot-causing bacteria isolated from tomatoes in Iğdır Plain. We used conventional biochemical tests for initial identification, investigated their pathogenicity on tomatoes, and analyzed their cellular fatty acid composition (FAME analysis), and sequences.

MATERIALS AND METHODS

Collection of Plant Samples and Isolation of Bacteria

Surveys were conducted during the spring and autumn of 2021 and 2022 in tomato

fields located in Iğdır. Samples showing typical symptoms of bacterial spot were randomly collected from different parts of tomato plants (leaves and fruits) (Figure 1). Symptomatic plant parts were cut from plant using a sterile equipment, placed in polyethylene bags, and stored at 4°C until the isolation process.

Symptomatic plant parts were prepared for bacterial isolation. First, the diseased and healthy parts were separated from the washed samples using a scalpel. These pieces were then disinfected with sodium hypochlorite (2 minutes) and then rinsed with sterile water. In a sterilized mortar, the plant material was crushed in sterile distilled water to form a suspension. Subsequently, 100 microliters (µL) of the suspension were plated onto petri dishes containing Yeast extract–Dextrose–Calcium carbonate (YDC) medium. The petri dishes were incubated at 24±28 °C to promote bacterial growth. After 48 h incubation, distinct yellow, round, and mucoid colonies were isolated. All the bacterial strains were subjected to a hypersensitivity test using tobacco (*Nicotiana benthamiana)* plants and their Gram reactions was determined using 3% potassium hydroxide. Pure cultures of these bacteria were prepared and stored at -80 °C for further analysis (Schaad *et al.*, 2001).

Pathogenicity of Bacterial Strains on Tomato Plants

Pathogenicity assays were conducted following the method described by AL-Saleh (2011). Pathogenicity of bacterial strains listed in Table 1 was assessed in tomato plants using *Xanthomonas axonopodis* pv. *vesicatoria* strain BS-120 (Sunyar *et al.*, 2021) as a reference for comparison. In this study, we used hrpB gene sequences retrieved from GenBank for reference strains and various *Xanthomonas* species such as *Xanthomonas phaseoli* pv. *dieffenbachiae* (formerly *Xanthomonas axonopodis* pv. *dieffenbachiae*) strain X1708 (AY576628.1), *Xanthomonas hortorum* pv. *gardneri* strain ETH8 (KF994848), *X. euvesicatoria* pv. *perforans* (formerly *X. perforans*) strain ETH11 (KF994851), *Xanthomonas vesicatoria* strain ETH20 (KF994860), *Xanthomonas* sp. NI15 (KJ938623), *X. euvesicatoria* pv. *euvesicatoria* (formerly *X. euvesicatoria*) strain Xeu7 (KU315002), *X. euvesicatoria* pv. *perforans* (formerly *X. perforans*) strain ICMP-16690 (KU594480), *X. euvesicatoria* pv. *euvesicatoria* (formerly *X. euvesicatoria*) PhXeu-3 (MG657344), *X. euvesicatoria* pv. *euvesicatoria* (formerly *X. euvesicatoria*) strain 19_57_10a (MN824429) and *X. euvesicatoria* pv. *perforans* (formerly *X. perforans*) strain PJT 7 (OP820590). A popular local variety, cv. 'Süper domates', commonly grown in Iğdır, was chosen for this pathogenicity test. Bacteria were grown on YDC medium in petri dishes for 48 hours at 24±28°C. Plants

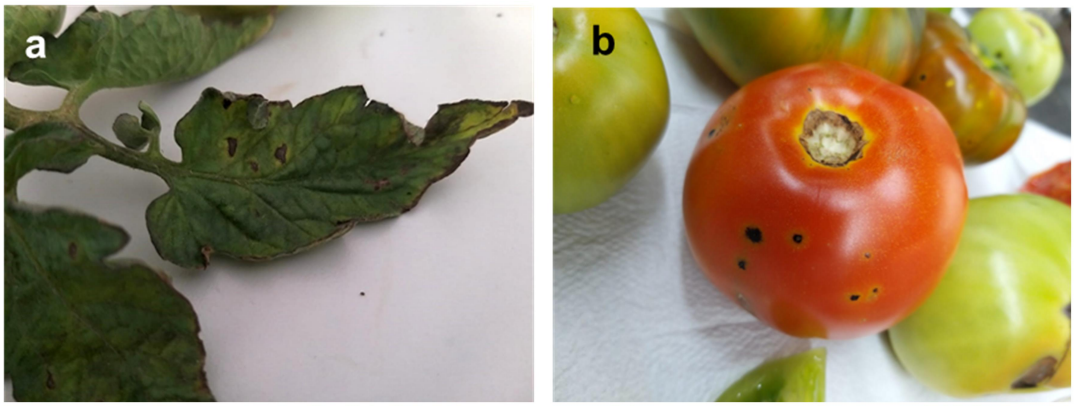

Figure 1. Field symptoms of tomato bacterial spot: (a) Necrotic lesions with yellowing margins on leaves, and (b) Bacterial spot symptoms on tomato fruit.

were inoculated with the bacteria or sterile water (negative control) and kept in the greenhouse for symptom observation. Disease severity was evaluated 21 days after inoculation. Disease symptoms on the leaves were evaluated using a scale of 1 to 5, adapted from Sahin and Miller (1998). The scale is as follows: (1) No signs of disease, (2) A few scattered, water-soaked lesions, (3) Numerous spots that have merged, with slight wilting of the plant, (4) Significant defoliation of leaves, and (5) Dead plant. Disease severity (%) was then calculated as a percentage using the following formula* developed by Towsend and Heuberger (1943):

$$* \text{ Disease severity (\%)} = \frac{\sum(n \times v)}{(z \times N)} \times 100$$

Where, n is the number of samples in the scale with different disease grades, v is the scale value, z is the highest scale value, and N is the total Number of samples.

Phenotypic Characteristics of Bacterial Strains and FAME Analysis

Further tests were conducted to identify the characteristics of the pathogenic bacteria, focusing on traits common to *Xanthomonas* species that infect tomatoes. These tests are as follows: Catalase assay was performed with 7% H_2O_2 solution and oxidase assay was performed using disks containing 1% tetra methyl-p-phenylendiamine dihydrochloride (Narayanasamy, 2001). The amylase activity of the strains was determined using Nutrient Agar (NA) medium containing 1% starch (Hélias *et al.*, 2012). These strains were analyzed using a gas chromatography system (Agilent 7890A GC System, MIDI Inc.) in combination with specialized software (Sherlock Version 6.1). This system generates a unique "fingerprint" based on the Fatty Acid profiles (FAME) of the bacteria. These fingerprints were then compared with reference library (RTSBA 6) to identify the most likely bacterial species (Sasser, 1990).

Molecular Diagnosis and Phylogenetic Analysis of Bacterial Strains

DNA extraction from 24-hour pure cultured bacteria was performed using commercial bacterial Genomic DNA Minipreps Kit (Bio Basic, Cat. No. BS423-50). The integrity of the DNA was validated by agarose gel electrophoresis and the concentration was measured with micro-volume spectrophometer (Maestrogen, Taiwan). To determine the species of *Xanthomanas*, we used the end-point Polymerase Chain Reaction (PCR) technique with the primer pairs previously described in the literature; Bs-XeF-Bs-XeR, Bs-XvF-Bs-XvR, Bs-XgF-Bs-XgR and Bs-XpF-Bs-XpR (Koenraadt *et al.*, 2009). Twenty-five microliters of the reaction mix were prepared with 2X Reaction Buffer (Thermo Scientific, Cat. No. EP0401), 0.1 mM dNTPs, 0.2 µM forward and reverse primers, 1 U Taq DNA polymerase (Thermo Scientific, Cat. No. EP0401), 1 mM Mg+2, 20 ng DNA and nuclease-free water. Thermal cycling was performed on SimpliAmp (Applied Biosystems, USA) instrument under the following conditions: initial denaturation at 95°C for 3 minutes and followed by 35 cycles of 95°C for 30 seconds denaturation, 57.4°C for 45 seconds annealing, 72°C for 1 minute elongation. PCR was finalized at 72°C for 10 minutes, a final elongation step. The PCR products were run on 2% agarose gel to control for the presence of amplicons.

RST65 and RST69 primer pair (Obradovic *et al.*, 2004) was used to sequence the ATP-dependent RNA helicase (*hrpB*) gene for phylogenetic analysis of the species. The reaction mixes and PCR condition were identical with the molecular diagnosis of pathogenic bacteria strains experiment. PCR products were directly sent to Macrogen Inc. (The Netherlands) for purification and both-direction Sanger dideoxy sequencing using

RST65 and RST69 primers. The DNA sequences were imported to Geneious Prime (20243.0.3) software suit for quality score check, trim, and obtain consensus sequences for quality checking, trimming the primer binding sites and obtaining the consensus sequences by assembling both directions reads. A phylogenetic tree was constructed with PAUP 4a using Maximum Parsimony approach (Heuristic search) (Swofford, 2003).

RESULTS

Establishment of a Bacterial Collection

We successfully isolated a total of ten *Xanthomonas* strains from 35 plant samples. Four strains were isolated from diseased tomato leaves, and the remaining six bacterial strains were obtained from the fruits of the plants. Details of these isolated strains are presented in the Table 1.

Phenotypic Characterization and Pathogenicity of Bacterial Strains

All ten strains isolated from tomato plants triggered a hypersensitive reaction in tobacco plants. These strains formed distinct yellow, circular, mucoid, and shiny colonies when grown on YDC medium. The results of biochemical teste are shown in the Table 1. All strains were Gram-negative bacteria with positive catalase activity, but lacked oxidase activity. The ability to break down starch (amylolytic activity) varied among the strains. Strains XCV7 showed strong amylolytic activity, whereas XCV1, XCV2, XCV3, XCV5 and XCV7/1 displayed positive result. The remaining strains XCVO, XCVOZ, XCVOZ1, and XCVOZ2 were negative for amylolytic activity. A gas chromatography system, called the Microbial Identification System, was used to analyze the fatty acid profiles of the ten strains. Based on these profiles, the strains were identified as *Xanthomonas campestris* pv. *vesicatoria* with a similarity index ranging from 61 to 78%. The details of the identification and similarity index can be found in the Table 2. In addition, these strains were subjected to pathogenicity tests (Figure 2). The disease severity values (%) obtained from the pathogenicity tests of the strains are given in the Table 1. All ten strains caused disease in cv. 'Süper domates' plants, as shown in Table 1. No symptoms appeared on negative control plants. While the reference strain caused severe disease in tomato plants (81% severity), the disease severity caused by the isolated strains ranged from 33.9 to 74%. XCV2 was the most virulent strain, causing 74% disease severity in tomatoes.

Table 1. The origin, biochemical, morphological, and pathogenicity traits of isolated *Xanthomonas* strains in this study.[a]

Strains	Location	Tissue	Year	Colony (YDC)	Gr	Hr	Ox	Ca	Am	Ds
XCV1	Melekli/Iğdır	Leaf	2021	Yellow-Circular	-	+	-	+	+	60.87
XCV2	Melekli/Iğdır	Fruit	2021	Yellow-Circular	-	+	-	+	+	74.00
XCV3	Melekli/Iğdır	Fruit	2021	Yellow-Circular	-	+	-	+	+	67.50
XCV5	Melekli/Iğdır	Fruit	2021	Yellow-Circular	-	+	-	+	+	53.65
XCV7	Tuzluca/Iğdır	Leaf	2021	Yellow-Circular	-	+	-	+	K+	32.98
XCV7/1	Tuzluca/Iğdır	Fruit	2021	Yellow-Circular	-	+	-	+	+	61.20
XCVO	Örüşmüş/Iğdır	Fruit	2022	Yellow-Circular	-	+	-	+	-	44.76
XCVOZ	Örüşmüş/Iğdır	Leaf	2022	Yellow-Circular	-	+	-	+	-	47.80
XCVOZ1	Örüşmüş/Iğdır	Fruit	2022	Yellow-Circular	-	+	-	+	-	33.91
XCVOZ2	Örüşmüş/Iğdır	Leaf	2022	Yellow-Circular	-	+	-	+	-	46.54

[a] Gr: Gram reaction, Hr: Hypersensitivity (Tobacco) test, Ox: Oxidase test, Ca: Catalase test, Am: Amylolytic activity (K^+-strong positive), Ds: Diseases severity (%), +: Positive, -: Negative

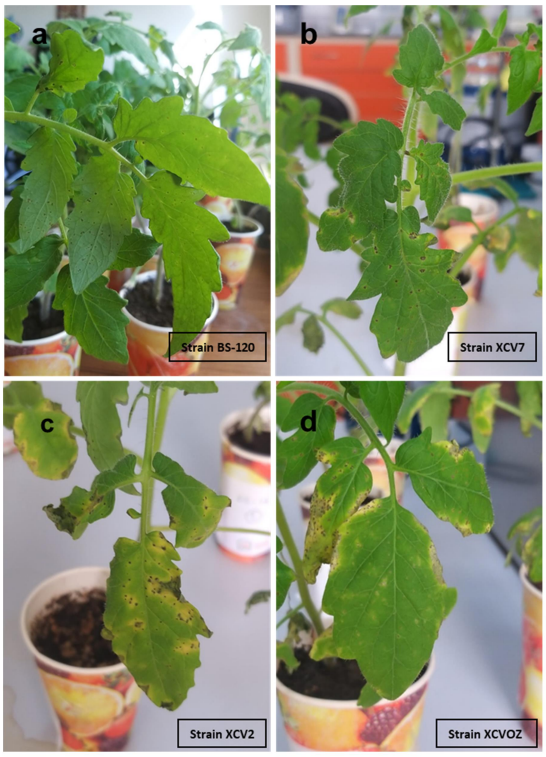

Figure 2. Symptoms induced on tomato plants by inoculations with *X. euvesicatoria* pv. *perforans* (strains XCV2-XCV7), *X. euvesicatoria* pv. *euvesicatoria* strain XCVOZ and *Xanthomonas axonopodis* pv. *vesicatoria* strain BS-120 (control +): (a) Circular water-soaked lesions on the leaves, (b) Initial symptoms of bacterial spot of tomato, (c) Advanced symptoms of bacterial spot of tomato, and (d) Necrotic lesions on the leaves surrounded by chlorotic margins.

Molecular Diagnosis and Phylogenetic Analysis of Bacterial Strains

Since the integrity and the concentrations of DNA extracts (varied between 28.74 to 78.16 ng μL^{-1}) were appropriate, we directly proceeded to downstream applications PCR and sequencing. PCR results showed that the Bs-XeF-Bs-XeR primer pair successfully amplified the related DNA region for XCVO, XCVOZ, XCVOZ1 and XCVOZ2 strains, whereas the other primers pair did not (Table 2).

The RST65 and RST69 primer pairs were used to amplify the *HrpB* gene region for sequencing. Sanger dideoxy sequencing was successful for all samples. The assembled and trimmed sequence quality scores varied from 90.7 to 98.7%, which is reliable for further phylogenetic analysis. Additionally, all newly generated sequences were deposited in GenBank (Table 2). We performed BLASTn search for each sample and obtained the best hit results.The alignment length of the total 20 samples was 237 base pair (bp).

The cladogram consisted of three main clades (orange, blue, and violet) and an outgroup (Figure 3). *X. phaseoli* pv. *dieffenbachiae* strain X1708 was placed as an outgroup to *X. euvesicatoria* pv. *perforans* and *X. euvesicatoria* pv. *euvesicatoria* samples as supposed. *Xanthomonas hortorum* pv. *gardneri* and *X. vesicatoria* were placed in the first clade (violet), *X. euvesicatoria* pv. *perforans* strains in the second (blue) clade, and *X. euvesicatoria* pv. *euvesicatoria* strains in the third clade (orange). The violet clade was

Table 2. Identification of bacterial strains based on FAME, Species-specific PCR and the sequencing of *hrpB* gene.[a]

Strains	FAME		Species-specific PCR				Identity based on *hrpB*		
	FSI (%)	FAME	a	b	c	d	Species	SI (%)	Accession[b]
XCV1	73	*X. campestris* pv. *vesicatoria*	-	-	-	-	*X. euvesicatoria* pv. *perforans*	100	PP505864
XCV2	78	*X. campestris* pv. *vesicatoria*	-	-	-	-	*X. euvesicatoria* pv. *perforans*	100	PP505863
XCV3	61	*X. campestris* pv. *vesicatoria*	-	-	-	-	*X. euvesicatoria* pv. *perforans*	100	PP505867
XCV5	68	*X. campestris* pv. *vesicatoria*	-	-	-	-	*X. euvesicatoria* pv. *perforans*	100	PP505868
XCV7	55	*X. campestris* pv. *vesicatoria*	-	-	-	-	*X. euvesicatoria* pv. *perforans*	100	PP505870
XCV7/1	76	*X. campestris* pv. *vesicatoria*	-	-	-	-	*X. euvesicatoria* pv. *perforans*	100	PP505869
XCVO	60	*X. campestris* pv. *vesicatoria*	+	-	-	-	*X. euvesicatoria* pv. *euvesicatoria*	100	PP505865
XCVOZ	57	*X. campestris* pv. *vesicatoria*	+	-	-	-	*X. euvesicatoria* pv. *euvesicatoria*	100	PP505861
XCVOZ1	72	*X. campestris* pv. *vesicatoria*	+	-	-	-	*X. euvesicatoria* pv. *euvesicatoria*	100	PP505866
XCVOZ2	69	*X. campestris* pv. *vesicatoria*	+	-	-	-	*X. euvesicatoria* pv. *euvesicatoria*	100	PP505862

[a] FAME: Fatty Acid Methyl Ester, FSI: FAME Similarity Index; Species-specific Primers: Bs-XeF/XeR (a), Bs-XpF/XpR (b), Bs-XvF/XvR (c), Bs-XgF/XgR (d); **SI:** Similarity Index.
[b] NCBI accession numbers of the sequences generated with the present study. +: Positive, -: Negative.

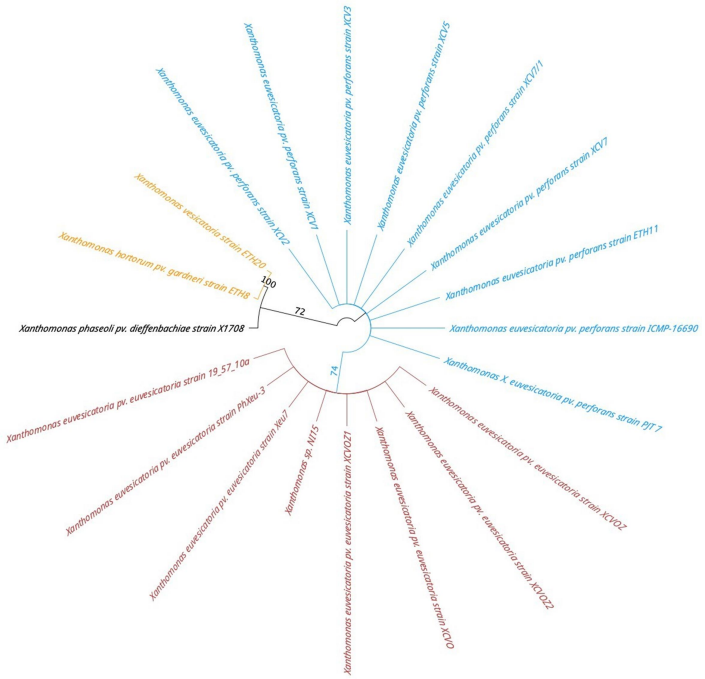

Figure 3. Phylogenetic relationships among 20 strains of the *Xanthomonas* species complex were obtained with sequence analysis using *HrpB* gene region by Maximum Parsimony approach. The bootstrap (1,000 replicates) consensus values were placed on the branches.

placed as sister group to the orange and blue clades.

DISCUSSION

Various bacterial pathogens can infect tomato plants at different stages of growth, causing a range of diseases (Lin and Wang, 2010). This study focused on the presence of bacterial spot disease in tomato plants from the Iğdır Plain. Ten bacterial strains were obtained from these tomato plants, and all strains exhibited varying degrees of virulence (Table 1), causing bacterial spot symptoms on tomato plants. *Xanthomonas* species possess various virulence factors, including adhesins for plant attachment, flagella and fimbriae for motility and adhesion, and exopolysaccharides and lipopolysaccharides for interaction with the plant environment. Additionally, secretion systems inject effector molecules that manipulate plant processes, while extracellular degrading enzymes break down plant cell walls for nutrient access. This coordinated deployment of virulence factors empowers *Xanthomonas* to overcome plant resistance and establish disease (An *et al.,* 2020). While these virulence factors play a crucial role in *Xanthomonas* species colonization and infection of tomatoes, further investigation is needed to pinpoint additional critical factors specific to our strains. Tomato bacterial spot is caused by a group of related bacteria within the *Xanthomonas* genus (Jones *et al.*, 2004). To identify and differentiate among these specific bacteria, researchers now rely on a combination of molecular and biochemical techniques (Araújo *et al.*, 2012). While conventional methods, such as biochemical tests, have been used to differentiate between *Xanthomonas* species (Jones *et al*., 1998), the emergence of new unique strains can challenge this approach and lead to misidentification.

FAME analysis can be used as a preliminary screening tool to differentiate between broad groups of bacteria based on their overall fatty acid makeup (Gilbride, 2014; Kunitsky *et al.*, 2006). FAME analysis was used to characterize the fatty acid profiles of the bacterial strains in this study. All bacterial strains were identified as *X. campestris* pv. *vesicatoria* by FAME with a similarity index ranging from 0.55-0.78. In the 1990s, researchers identified two distinct groups within *X. campestris* pv. *vesicatoria*: group A and group B (Stall *et al.*, 1994; Vauterin *et al.*, 1990). Group A strains were uniformly negative for amylolytic and pectolytic activity, while group B strains were strongly active in both (Bouzar *et al.*, 1994). Our study found similar variation. Six strains displayed amylolytic activity, which is consistent with group B and *X. euvesicatoria* pv. *perforans* strains (Jones *et al.*, 2004). However, the remaining four strains did not exhibit amylolytic activity, consistent with group A and *X. euvesicatoria* pv. *euvesicatoria*. While FAME analysis and biochemical tests provided valuable insights, these findings suggest they may not be sufficient for definitively identifying all four tomato-associated *Xanthomonas* pathogens. However, FAME analysis shows promise as a rapid pre-screening tool to identify potentially pathogenic *Xanthomonas* strains in tomatoes. For conclusive identification, complementary techniques might still be necessary.

A more reliable approach for diagnosing *Xanthomonas*-caused plant diseases utilizes species-specific primers (Pan *et al.*, 1999; Suk Park *et al.*, 2006). These primers target specific DNA sequences that are unique to each *Xanthomonas* species, allowing for more accurate identification. Previously, methods like the RST 65/69 primers (Leite *et al.*, 1995) were used, but they amplified DNA from a broader group of *Xanthomonas* species. This broader targeting could lead to misidentification, as these primers produced the same sized amplicon not only for tomato bacterial spot strains but also for other unrelated *Xanthomonas* species. Researchers have developed numerous sets of primers specifically designed to identify different *Xanthomonas* species that cause bacterial

spot disease (Araújo *et al.*, 2012; Astua-Monge *et al.*, 2000; Cuppels *et al.*, 2006; Koenraadt *et al.*, 2009; Moretti *et al.*, 2009). Species–specific primer pairs (BS-XeF/BS-XeR, BS-XvF/BS-XvR, BS-XgF/BS-XgR and BS-XpF/BS-XpR) designed by Koenraadt *et al.* (2009) were used to differentiate these tomato-associated *Xanthomonas* species in this study. PCR using the BS-XeF/BS-XeR primer set identified the expected 173-bp amplicon specific for *X. euvesicatoria* pv. *euvesicatoria* in only four strains. None of the remaining strains produced amplicons when using this primer set. None of the strains were amplified with any other species-specific primer sets tested. PCR using the BS-XeF/BS-XeR primer set and the subsequent sequencing of the *hrpB* gene yielded concordant results. Four out of ten tomato strains were identified as *X. euvesicatoria* pv. *euvesicatoria* using both methods, while the remaining six strains clustered with *X. euvesicatoria* pv. *perforans* (Figure 3).

However, *X. euvesicatoria* pv. *perforans* strains in this study were not detected using the species-specific primer pair Bs-XpF/Bs-XpR. Osdaghi *et al.* (2017) reported similar findings with this primer set. The limited detection of *X. euvesicatoria* pv. *perforans* in this study using the Bs-XpF/Bs-XpR primers aligns with observations by Osdaghi *et al.* (2017) who suggested that these primers might not be sensitive enough to capture the full global diversity of *X. euvesicatoria* pv. *perforans*. This is further supported by the fact that all tested strains originated from a single location near the Iranian border in Iğdır, Turkey. A wider geographical range of strains might be necessary to comprehensively evaluate the effectiveness of these primers for *X. euvesicatoria* pv. *perforans* detection.

Despite extensive research on pathogenic *Xanthomonas* in tomatoes in Turkey, researchers have not discriminated against exact species using molecular techniques for the current four species (Aysan and Sahin, 2003; Basim *et al.*, 2004; Mirik and Aysan, 2009). A study performed by Eryigit (2016) in Turkey tested ten *Xanthomonas* strains. Eight of these strains were identified as *X. euvesicatroia* using both biochemical tests and species-specific PCR analyses. The remaining two strains were identified as *X. euvesicatoria* pv. *perforans* solely through biochemical testing. According to recent whole-genome sequencing study, two bacterial spot pathogens have been reported in Turkey: *X. euvesicatoria* pv. *euvesicatoria* on peppers and *X. euvesicatoria* pv. *perforans* on tomatoes, peppers, and eggplants (Subedi *et al.*, 2023). This study confirms the findings of previous studies on bacterial spot pathogens. Although bacterial spot disease was reported in Iğdır (Sunyar *et al.*, 2021), this is the first study to identify *X. euvesicatoria* pv. *euvesicatoria* and *X. euvesicatoria* pv. *perforans* as the specific bacteria causing the disease in tomatoes grown on the Iğdır Plain.

CONCLUSIONS

This study investigated the causal agents of bacterial spot symptoms on tomatoes cultivated in the Iğdır Plain, Turkey. Ten *Xanthomonas* strains, isolated from these symptomatic plants, underwent pathogenicity testing and were subsequently identified using molecular and biochemical techniques. As a result, it was determined that *X. euvesicatoria* pv. *euvesicatoria* and *X. euvesicatoria* pv. *perforans* strains were the causal agents of bacterial spot disease in tomatoes grown in the Iğdır Plain.

REFERENCES

1. AL-Saleh, M. A. 2011. Pathogenic Variability among Five Bacterial Isolates of *Xanthomonas campestris* pv. *vesicatoria*, Causing Spot Disease on Tomato and Their Response to Salicylic Acid. *J. Saudi Soc. Agric. Sci.*, **10(1)**: 47–51.
2. An, S. Q., Potnis, N., Dow, M., Vorhölter, F. J., He, Y. Q., Becker, A., Teper, D., Li, Y.,

Wang, N., Bleris, L. and Tang, J. L. 2020. Mechanistic Insights into Host Adaptation, Virulence and Epidemiology of the Phytopathogen *Xanthomonas*. *FEMS Microbiol. Rev.,* **44(1):** 1–32.

3. Araújo, E. R., Costa, J. R., Ferreira, M. A. S. V. and Quezado-Duval, A. M. 2012. Simultaneous Detection and Identification of the *Xanthomonas* Species Complex Associated with Tomato Bacterial Spot Using Species-Specific Primers and Multiplex PCR. *J. Appl. Microbiol.,* **113(6):** 1479–1490.
4. Astua-Monge, G., Minsavage, G. V., Stall, R. E., Davis, M. J., Bonas, U. and Jones, J. B. 2000. Resistance of Tomato and Pepper to T3 Strains of *Xanthomonas campestris* pv. *vesicatoria* is Specified by a Plant-Inducible Avirulence Gene. *Mol. Plant Microbe Interact.,* **13(9):** 911–921.
5. Aydın, T. and Çelik, M. A. 2019. Altitudinal Zone L and Use Changes in Iğdir Plain Using Overlay Analysis Combined with Remote Sensing Methods. *J. Remote Sens. GIS*, **8(263)**: 1–8.
6. Aysan, Y. and Sahin, F. 2003. Occurrence of Bacterial Spot Disease, Caused by *Xanthomonas axonopodis* pv. *vesicatoria*, on Pepper in the Eastern Mediterranean Region of Turkey. *Plant Pathol.*, **52(6):** 781–781.
7. Bashan, Y., Azaizeh, M., Diab, S., Yunis, H. and Okon, Y. 1985. Crop Loss of Pepper Plants Artificially Infected with *Xanthomonas campestris* pv. *vesicatoria* in Relation to Symptom Expression. *J. Crop Prot.*, **4(1):** 77–84.
8. Basim, H., Basim, E., Jones, J. B., Minsavage, G. V. and Dickstein, E. R. 2004. Bacterial Spot of Tomato and Pepper Caused by *Xanthomonas axonopodis* pv. vesicatoria in the Western Mediterranean Region of Turkey. *Plant Dis.*, **88(1):** 85–85.
9. Bouzar, H., Jones, J. B., Minsavage, G. V., Stall, R. E. and Scott, J. W. 1994. Proteins Unique to Phenotypically Distinct Groups of *Xanthomonas campestris* pv. *vesicatoria* Revealed by Silver Staining. *Phytopathology*, **84(1):** 39–44.
10. Büttner, D. and Bonas, U. 2010. Regulation and Secretion of *Xanthomonas* Virulence Factors. *FEMS Microbiol. Rev.*, **34(2):** 107–133.
11. Constantin, E. C., Cleenwerck, I., Maes, M., Baeyen, S., Van Malderghem, C., De Vos, P. and Cottyn, B. 2016. Genetic Characterization of Strains Named as *Xanthomonas axonopodis* pv. *dieffenbachiae* Leads to a Taxonomic Revision of the *X. axonopodis* Species Complex. *Plant Pathol.*, **65(5)**: 792–806.
12. Cuppels, D. A., Louws, F. J. and Ainsworth, T. 2006. Development and Evaluation of PCR-Based Diagnostic Assays for the Bacterial Speck and Bacterial Spot Pathogens of Tomato. *Plant Dis.*, **90(4)**: 451–458.
13. EFSA Panel on Plant Health. 2014. Scientific Opinion on the Pest Categorisation of *Xanthomonas campestris* pv. *vesicatoria* (Doidge) Dye. *EFSA J.*, **12(6)**: 1-26.
14. EPPO. 2013. PM 7/110 (1) Xanthomonas spp. (Xanthomonas euvesicatoria, Xanthomonas gardneri, Xanthomonas perforans, Xanthomonas vesicatoria) Causing Bacterial Spot of Tomato and Sweet Pepper. EPPO Bull., **43(1)**: 7–20.
15. Eryigit, G. 2016. Classical and Molecular Diagnostic of *Xanthomonad* Species Causing Bacterial Spot on Tomato and Pepper. MSc. Thesis, Graduate School of Natural and Applied Science, Faculty of Agriculture, Ege University, 95 PP. (in Turkish).
16. FAO (Food and Agriculture Organization). 2022. *Production of Tomatoes: Top 10 Producers*. https://www.fao.org/faostat/en/#data/QCL/visualize, Accessed 27 March 2023
17. Gilbride, K. 2014. Molecular Methods for the Detection of Waterborne Pathogens. In: "*Waterborne Pathogens: Detection Methods and Applications",* (Ed.): Bridle, H. Academic Press, Elsevier B. V.; London, UK, PP. 231–290.
18. Hélias, V., Hamon, P., Huchet, E., Wolf, J. V. D., and Andrivon, D. 2012. Two New Effective Semiselective Crystal Violet Pectate Media for Isolation of *Pectobacterium* and *Dickeya*. *Plant Pathol.*, **61(2)**: 339–345.
19. Jones, J. B., Jones, J. P., Stall, R. E. and Zitter, T. A. 1991. *Compendium of Tomato Diseases*. American Phytopathological Society Press, St. Paul, 73 PP.
20. Jones, J. B., Lacy, G. H., Bouzar, H., Stall, R. E. and Schaad, N. W. 2004. Reclassification of the *Xanthomonads* Associated with Bacterial Spot Disease of Tomato and Pepper. *Syst. Appl. Microbiol.*, **27(6):** 755–762.
21. Jones, J. B., Stall, R. E. and Bouzar, H. 1998. Diversity among *Xanthomonads* Pathogenic

on Pepper and Tomato. *Annu. Rev. Phytopathol.,* **36:** 41–58.
22. Jones, J. B., Zitter, T. A., Momol, T. M. and Miller, S. A. 2013. *Compendium of Tomato Diseases and Pests*. Second Edition, APS Press, St. Paul, MN.
23. Kayaaslan, Z., Belgüzar, S., Yanar, Y. and Mirik, M. 2023. Epidemiology of *Xanthomonas euvesicatoria* in Tokat Province. *Agronomy,* **13(3):** 1-9.
24. Kebede, M., Timilsina, S., Ayalew, A., Admassu, B., Potnis, N., Minsavage, G. V., Goss, E. M., Hong, J. C., Strayer, A., Paret, M., Jones, J. B. and Vallad, G. E. 2014. Molecular Characterization of *Xanthomonas* Strains Responsible for Bacterial Spot of Tomato in Ethiopia. *Eur. J. Plant Pathol.*, **140(4)**: 677–688.
25. Koenraadt, H., Van Betteray, B., Germain, R., Hiddink, G., Jones, J. B., Oosterhof, J., Rijlaarsdam, A., Roorda, P. and Woudt, B. 2009. Development of Specific Primers for the Molecular Detection of Bacterial Spot of Pepper and Tomato. *Acta Hortic.*, **808:** 99–102.
26. Kunitsky, C. J., Osterhout, G. and Sasser, M. 2006. Identification of Microorganisms Using Fatty Acid Methyl Ester (FAME) Analysis and the MIDI Sherlock Microbial Identification System. *Encycl. Rapid Microbial. Methods*, **3:** 1–18.
27. Leite, R. P., Jones, J. B., Somodi, G. C., Minsavage, G. V. and Stall, R. E. 1995. Detection of *Xanthomonas campestris* pv. *vesicatoria* Associated with Pepper and Tomato Seed by DNA Amplification. *Plant Dis.*, **79(9):** 917–922.
28. Leyns, F., De Cleene, M., Swings, J. G. and De Ley, J. 1984. The Host Range of the Genus *Xanthomonas*. *Bot. Rev.,* **50(3):** 308–356.
29. Lin, C. H. and Wang, J. F. 2010. Managing Bacterial Diseases of Tomato. In: "*Safer Tomato Production Techniques: A Field Guide for Soil Fertility and Pest Management"*, (Ed.): Srinivasan, R. AVRDC - The World Vegetable Center, **10(7):** 61–68.
30. Mirik, M. and Aysan, Y. 2009. Detection of *Xanthomonas axonopodis* pv. *vesicatoria* in Naturally Infected Pepper Seeds in Turkey. *J. Plant Pathol*., **91(2):** 433–436.
31. Moretti, C., Amatulli, M. T. and Buonaurio, R. 2009. PCR-Based Assay for the Detection of *Xanthomonas euvesicatoria* Causing Pepper and Tomato Bacterial Spot. *Lett. Appl. Microbiol*., **49(4):** 466–471.
32. Morinière, L., Burlet, A., Rosenthal, E. R., Nesme, X., Portier, P., Bull, C. T., Lavire, C., Fischer-Le Saux, M. and Bertolla, F. 2020. Clarifying the Taxonomy of the Causal Agent of Bacterial Leaf Spot of Lettuce through a Polyphasic Approach Reveals that *Xanthomonas cynarae* Trébaol *et al.* 2000 emend. Timilsina *et al.* 2019 Is a Later Heterotypic Synonym of *Xanthomonas hortorum* Vauterin *et al.* 1995. *Syst. Appl. Microbiol.,* **43(4):** 1-16.
33. Narayanasamy, P. 2001. *Plant Pathogen Detection and Disease Diagnosis.* 2nd Edition, CRP Press. 544 PP.
34. Obradovic, A., Mavridis, A., Rudolph, K., Janse, J. D., Arsenijevic, M., Jones, J. B., Minsavage, G. V. and Wang, J. F. 2004. Characterization and PCR-Based Typing of *Xanthomonas campestris* pv. *vesicatoria* From Peppers and Tomatoes in Serbia. *Eur. J. Plant Pathol.*, **110(3)**: 285–292.
35. Osdaghi, E., Jones, J. B., Sharma, A., Goss, E. M., Abrahamian, P., Newberry, E. A., Potnis, N., Carvalho, R., Choudhary, M., Paret, M. L., Timilsina, S. and Vallad, G. E. 2021. A Centenary for Bacterial Spot of Tomato and Pepper. *Mol. Plant Pathol.,* **22(12):** 1500–1519.
36. Osdaghi, E., Taghavi, S. M., Hamzehzarghani, H., Fazliarab, A. and Lamichhane, J. R. 2017. Monitoring the Occurrence of Tomato Bacterial Spot and Range of the Causal Agent *Xanthomonas perforans* in Iran. *Plant Pathol.*, **66(6):** 990–1002.
37. Padmanabhan, P., Cheema, A. and Paliyath, G. 2016. Solanaceous Fruits Including Tomato, Eggplant, and Peppers. In: "*Encyclopedia of Food and Health"*, (Eds.): Cabellero, B., Finglas, P. and Toldra, F. 1st Edition, Oxford: Academic Press, PP. 24–32.
38. Pan, Y. B., Grisham, M. P., Burner, D. M., Legendre, B. L. and Wei, Q. 1999. Development of Polymerase Chain Reaction Primers Highly Specific for *Xanthomonas albilineans*, the Causal Bacterium of Sugarcane Leaf Scald Disease. *Plant Dis.*, **83(3):** 218–222.
39. Potnis, N., Timilsina, S., Strayer, A., Shantharaj, D., Barak, J. D., Paret, M. L., Vallad, G. E. and Jones, J. B. 2015. Bacterial Spot of Tomato and Pepper: Diverse *Xanthomonas* Species with a Wide Variety of

Virulence Factors Posing a Worldwide Challenge. *Mol. Plant Pathol.,* **16(9):** 907–920.
40. Sahin, F. and Miller, S. A. 1998. Resistance in *Capsicum pubescens* to *Xanthomonas campestris* pv. *vesicatoria* Pepper Race 6. *Plant Dis.*, **82(7):** 794–799.
41. Sasser, M. 1990. *Bacterial Identification by Gas Chromatographic Analysis of Fatty Acid Methyl Esters (GC-FAME).* MIDI Technical Note #101, PP. 1–6. https://gcms.cz/labrulez-bucket-strapi-h3hsga3/paper/MIS_Technote_101.pdf
42. Schaad, N. W., Jones, J. B. and Chun, W. 2001. Laboratory Guide for Identification of Plant Pathogenic Bacteria. *Biologia Plantarum,* **44**: 546.
43. Stall, R. E., Beaulieu, C., Egel, D., Hodge, N. C., Leite, R. P., Minsavage, G. V., Bouzar, H., Jones, J. B., Alvarez, A. M. and Benedict, A. A. 1994. Two Genetically Diverse Groups of Strains Are Included in *Xanthomonas campestris* pv. *vesicatoria*. *Int. J. Syst. Bacteriol.*, **44(1):** 47–53.
44. Subedi, A., Kara, S., Aysan, Y., Minsavage, G. V., Timilsina, S., Roberts, P. D., Goss, E. M. and Jones, J. B. 2023. Draft Genome Sequences of 11 *Xanthomonas* Strains Associated with Bacterial Spot Disease in Turkey. *Access Microbiol.*, **5(6):** 1-11.
45. Suk Park, D., Wook Hyun, J., Jin Park, Y., Sun Kim, J., Wan Kang, H., Ho Hahn, J. and Joo Go, S. 2006. Sensitive and Specific Detection of *Xanthomonas axonopodis* pv. *citri* by PCR Using Pathovar Specific Primers Based on hrpW Gene Sequences. *Microbiol. Res.*, **161(2)**: 145–149.
46. Sunyar, B., Dönmez, M. F. and Çoruh, İ. 2021. Iğdır'da Domates (*Solanum lycopersicon* L.)'te Hastalığa Neden Olan Bakterilerin Izolasyonu ve Tanısı. *J. Agriculture*, **4(2):**108–129.
47. Swofford, D. L. 2002. PAUP: Phylogenetic Analysis Using Parsimony (and Other Methods), Version 4.0 Beta 10. Sinauer Associates, Sunderland.
48. Towsend, G. R. and Heuberger, J. W. 1943. Methods for Estimating Losses Caused by Disease in Fungicide Experiments. *Plant Dis. Rep.*, **27:** 340-343.
49. Türkiye İstatistik Kurumu. 2022. *Bitkisel Üretim İstatistikleri: Domates Üretimi*. https://data.tuik.gov.tr/Kategori/GetKategori?p=tarim-111 anddil=1, Accessed 27 March 2023. (in Turkish)
50. Vauterin, L., Swings, J., Kersters, K., Gillis, M., Mew, T. W., Schroth, M. N., Palleroni, N. J., Hildebr and, D. C., Stead, D. E., Civerolo, E. L., Hayward, A. C., Maraite, H., Stall, R. E., Vidaver, A. K. and Bradbury, J. F. 1990. Towards an Improved Taxonomy of *Xanthomonas*. *Int. J. Syst. Bacteriol.*, **40(3)**: 312–316.
51. Yaltı, S. and Aksu, H. 2019. Drought Analysis of Iğdır Turkey. *Turk. J. Agric. Food Sci. Technol.*, **7(12)**: 2227–2232.
52. Young, J. M., Park, D. C., Shearman, H. M. and Fargier, E. 2008. A Multilocus Sequence Analysis of the Genus *Xanthomonas*. *Syst. Appl. Microbiol.*, **31(5):** 366–377.

شناسایی عوامل ایجاد کننده لکه های باکتریایی در مزارع گوجه فرنگی در دشت اژدر (Iğd r) (ترکیه)

مصطفی آکبابا، مسعود فیگن دونمز، و کان هورکان

چکیده

لکه‌های باکتریایی گوجه‌فرنگی که یک بیماری جدی است و عملکرد گوجه‌فرنگی را در ترکیه و بسیاری از کشورهای دیگر به میزان قابل توجهی کاهش می‌دهد. این پژوهش در دشت اژدر، با هدف شناسایی باکتری هایی که عامل بیماری لکه ای در گوجه فرنگی هستند انجام شد. بوته های گوجه فرنگی علامت دار از مزارع

داخل دشت برای جداسازی باکتری جمع آوری شد. ده سویه باکتری متعلق به جنس Xanthomonas از قسمت های مختلف (برگ و میوه) گیاه گوجه فرنگی جدا شد. از طریق یک سری تجزیه و تحلیل شامل ارزیابی های بیماری زایی، سنجش های بیوشیمیایی، پروفایل FAME، PCR با استفاده از پرایمرهای خاص گونه، و تجزیه و تحلیل فیلوژنتیکی توالی های ژن HrpB، سویه ها به طور قطعی به عنوان Xanthomonas euvesicatoria pv euvesicatoria وX. euvesicatoria pv *perforans*شناسایی شدند. با توجه به نتایج ما، FAME در تشخیص این دو گونه (X. euvesicatoria pv. euvesicatoria و X. euvesicatoria pv. perforans) موثر نبود. علاوه بر این، تنها پرایمر BS-XeF/BS-XeR توانست X.euvesicatoria pv را شناسایی کند و سایر آغازگرها، یعنی Bs-XpF/Bs-XpR قادر به تشخیص برخی از سویه های باکتریایی نبودند.تا آنجا که میدانیم، در حالی که بیماری لکه باکتریایی در مطالعات قبلی در دشت اژدر گزارش شده بود، پژوهش حاضر نشان دهنده شناسایی پیشگام X. euvesicatoria pv وX. perforans euvesicatoria pv به عنوان عوامل دقیق آن بیماری در گوجه فرنگی های کشت شده در این منطقه هستند. افزون بر این، X. euvesicatoria pv ، perforans. سویه XCV2 به عنوان خطرناک ترین سویه در این مطالعه شناسایی شد. میزان شدت آن در گوجه فرنگی ۷۴% بود (کولتیوار 'Sü per domates').