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

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ABSTRACT

The bacterial spot of tomatoes, a serious disease, significantly reduces tomato yields in Türkiye and many other countries. In Iğdır Plain, this study was aimed to characterize bacteria that are causal agents of bacterial 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 to, 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 within the Iğdır Plain. Additionally, *X. euvesicatoria* pv. *perforans* strain XCV2 was identified as the most virulent strain in this study. It caused a severe disease in tomato (cv. 'Süper domates') plants, with a severity rate of 74%.

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 reach approximately 186 million tons by 2022. Türkiye, with an annual production of approximately 13 million tons, ranks third in global tomato production

36 after China and India (Food and Agriculture Organization, 2022).

37 The Iğdır Plain is a low-lying area (between 800-900 meters above sea level) in eastern Turkey, 38 bordering Armenia, Azerbaijan and Iran (Aydın and Çelik, 2019). The surrounding mountains 39 create a unique microclimate with favorable temperatures for agriculture (Yaltı and Aksu, 40 2019). This allows for a diverse range of crops to be grown there, including tomatoes. Tomatoes 41 are the leading vegetable crop on the Iğdır Plain, boasting an annual yield of 35,217 tons 42 (Türkiye İstatistik Kurumu, 2022).

43 *Xanthomonas* is a genus of Gram-negative bacteria that includes several important plant 44 pathogens. These bacteria are obligate aerobes that obtain their energy through 45 chemoorganotrophy (Leyns *et al.*, 1984). A significant proportion of *Xanthomonas* species 46 exhibit plant pathogenic properties, causing infections in a wide range of monocotyledonous 47 and dicotyledonous crops (Büttner and Bonas, 2010). These include economically important 48 food crops such as tomato, and pepper (Kebede *et al.*, 2014; Potnis *et al.*, 2015). Tomato 49 bacterial spot is caused by a complex of *Xanthomonas* species, primarily *X. euvesicatoria*, *X.* 50 *vesicatoria*, *X. perforans*, and *X. gardneri* (Jones *et al.*, 2004). Recently, *X. euvesicatoria* and 51 *X. perforans* are now reclasified two pathovars of the same species, *X. euvesicatoria* pv. 52 *euvesicatoria (*formerly *X. euvesicatoria)* and *X. euvesicatoria* pv. *perforans (*formerly *X.* 53 *perforans)*, respectively (Constantin *et al.*, 2016). Meanwhile, *X. gardneri* has been reclassified 54 as *X. hortorum* pv. *gardneri* (Morinière *et al.*, 2020). Only *X. vesicatoria* remains the same 55 position (Osdaghi *et al.*, 2021). These four *Xanthomonas* species cause significant economic 56 losses on tomato production in both field and greenhouse settings and remain major limiting 57 factors for tomato production (EPPO, 2013). Tomato bacterial spot disease causal agents exhibit 58 a global distribution, affecting all aerial parts of the plant (Potnis *et al.*, 2015). Infected seeds, 59 volunteer crop plants, and diseased plant debris are the primary sources of inoculum for tomato 60 bacterial spot. The bacteria can be dispersed by rain and/or sprinkler irrigation droplets driven 61 by the wind within fields and from nearby areas (Jones *et al.*, 1991). Symptoms of disease 62 appear on various plant organs, including leaves, fruits, and stems (Jones *et al.*, 2013). Disease- 63 caused yield reduction is a consequence of a diminished photosynthetic leaf area, dropped buds 64 and flowers, and decreased commercial fruit quality. Severe infections can cause direct fruit 65 yield losses of 23 to 44%. Additionally, infected plants shed leaves, exposing fruits to damaging 66 sunlight and causing sunscald, leading to further indirect losses (Bashan *et al.*, 1985).

67 All four species are on a "high risk" list maintained by the European and Mediterranean Plant 68 Protection Organization (EPPO) with unique codes: XANTEU, XANTGA, XANTPF, and

69 XANTVE. They are classified as EPPO category A2 (no. 157) and are considered a significant
70 threat within the European Union (EU Annex II/A2). Hence, they are under strict quarantine
71 control and zero tolerance all over the worldwide (EFSA Panel on Plant Health, 2014; EPPO,
72 2013). Bacterial spot disease has been reported in many pepper and tomato producing areas in
73 Türkiye (Kayaaslan *et al.*, 2023).
74 Researchers have used species-specific primers to identify *Xanthomonas* species causing
75 bacterial spot (Koenraadt *et al.*, 2009). Also, analysis of the partial *hrpB* gene sequence was
76 considered a valuable tool for differentiating between *Xanthomonas* species at the species level
77 (Obradovic *et al.*, 2004; Young *et al.*, 2008). By combining these two methods – species-
78 specific primers and partial *hrpB* gene sequencing – researchers can achieve highly accurate
79 identification of the four *Xanthomonas* species known to cause bacterial spot disease in
80 tomatoes. No previous study has characterized the bacterial species on tomato in Iğdır Plain.
81 Thus, this study focused on characterizing bacterial spot-causing bacteria isolated from
82 tomatoes in Iğdır Plain. We used conventional biochemical tests for initial identification,
83 investigated their pathogenicity on tomatoes, and analyzed their cellular fatty acid composition
84 (FAME analysis), and sequences.
85
86 **MATERIALS AND METHODS**
87 **Collection of plant samples and isolation of bacteria**
88 Surveys were conducted during the spring and autumn of 2021 and 2022 in tomato fields
89 located in Iğdır. Samples showing typical symptoms of bacterial spot were r andomly collected
90 from different parts of tomato plants (leaves and fruits) **(Figure 1).** Symptomatic plant parts
91 were cut from plant using a sterile equipment, placed in polyethylene bags, and stored at 4 °C
92 until the isolation process.

93
94 **Figure 1.** Field symptoms of tomato bacterial spot: (a) necrotic lesions with yellowing margins on leaves, (b)
95 bacterial spot symptoms on tomato fruit.

96
97 Symptomatic plant parts were prepared for bacterial isolation. First, the diseased and healthy
98 parts were separated from the washed samples using a scalpel. These pieces were then
99 disinfected with sodium hypochlorite (2 min.) and then rinsed with sterile water. In a sterilized
100 mortar, the plant material was crushed in sterile distilled water to form a suspension.
101 Subsequently, 100 microliters (μl) of the suspension were plated onto petri dishes containing
102 yeast extract–dextrose–calcium carbonate (YDC) medium. The petri dishes were incubated at
103 24±28°C to promote bacterial growth. After 48 h incubation, distinct yellow, round, and mucoid
104 colonies were isolated. All the bacterial strains were subjected to a hypersensitivity test using
105 tobacco (*Nicotiana benthamiana)* plants and was determined their Gram reactions with using
106 3% potassium hydroxide. Pure cultures of these bacteria were prepared and stored at -80°C for
107 further analysis (Schaad *et al.*, 2001).
108
109 **Pathogenicity of bacterial strains on tomato plants**
110 Pathogenicity assays were conducted following the method described by AL-Saleh (2011).
111 Pathogenicity of bacterial strains listed in Table 1 was assessed in tomato plants using
112 *Xanthomonas axonopodis* pv. *vesicatoria* strain BS-120 (Sunyar *et al.*, 2021) as a reference for
113 comparison. In this study used hrpB gene sequences retrieved from GenBank for reference
114 strains and various *Xanthomonas* species such as *Xanthomonas phaseoli* pv. *dieffenbachiae*
115 (formerly *Xanthomonas axonopodis* pv. *dieffenbachiae*) strain X1708 (AY576628.1),
116 *Xanthomonas hortorum* pv. *gardneri* strain ETH8 (KF994848), *X. euvesicatoria* pv. *perforans*
117 (formerly *X. perforans*) strain ETH11 (KF994851), *Xanthomonas vesicatoria* strain ETH20
118 (KF994860), *Xanthomonas* sp. NI15 (KJ938623), *X. euvesicatoria* pv. *euvesicatoria* (formerly
119 *X. euvesicatoria*) strain Xeu7 (KU315002), *X. euvesicatoria* pv. *perforans* (formerly *X.*
120 *perforans*) strain ICMP-16690 (KU594480), *X. euvesicatoria* pv. *euvesicatoria* (formerly *X.*
121 *euvesicatoria*) PhXeu-3 (MG657344), *X. euvesicatoria* pv. *euvesicatoria* (formerly *X.*
122 *euvesicatoria*) strain 19_57_10a (MN824429) and *X. euvesicatoria* pv. *perforans* (formerly *X.*
123 *perforans*) strain PJT 7 (OP820590). A popular local variety, cv. 'Süper domates', commonly
124 grown in Iğdır, was chosen for this pathogenicity test. Bacteria were grown on YDC medium
125 in petri dishes for 48 hours at 24±28°C. Plants were inoculated with the bacteria or sterile water
126 (negative control) and kept in the greenhouse for symptom observation. Disease severity was
127 evaluated 21 days after inoculation. Disease symptoms on the leaves were evaluated using a
128 scale of 1 to 5 adapted from Sahin and Miller (1998): The scale is as follows: 1. no signs of
129 disease, 2. a few scattered, water-soaked lesions, 3. numerous spots that have merged, with

130 slight wilting of the plant, 4. significant defoliation of leaves, 5. dead plant. Disease severity
131 (%) was then calculated as a percentage using the formula* developed by Towsend and
132 Heuberger (1943): where, n is the number of samples in the scale with different disease grades,
133 v is the scale value, z is the highest scale value, and N is the total number of samples.

134 $$* \text{ Disease severity (\%)} = \frac{\sum(n \times v)}{(z \times N)} \times 100$$

135 **Phenotypic characteristics of bacterial strains and FAME analysis**

136 Further tests were conducted to identify the characteristics of the pathogenic bacteria, focusing
137 on traits common to *Xanthomonas* species that infect tomatoes. These tests are as follows:
138 Catalase assay was performed with 7% H_2O_2 solution and oxidase assay was performed using
139 disks containing 1% tetra methyl-p-phenylendiamine dihydrochloride (Narayanasamy, 2001).
140 The amylase activity of the strains was determined using Nutrient Agar (NA) medium
141 containing 1% starch (Hélias *et al.*, 2012). These strains were analyzed using a gas
142 chromatography system (Agilent 7890A GC System, MIDI Inc.) in combination with
143 specialized software (Sherlock Version 6.1). This system generates a unique "fingerprint" based
144 on the fatty acid profiles (FAME) of the bacteria. These fingerprints were then compared with
145 reference library (RTSBA 6) to identify the most likely bacterial species (Sasser, 1990).
146
147 **Molecular diagnosis and phylogenetic analysis of bacterial strains**

148 DNA extraction from 24-hour pure cultured bacteria was performed using commercial bacterial
149 Genomic DNA Minipreps Kit (Bio Basic, Cat. No. BS423-50). The integrity of the DNA was
150 validated by agarose gel electrophoresis and the concentration was measured with micro-
151 volume spectrophometer (Maestrogen, Taiwan). To determine the species of *Xanthomanas*, we
152 used the end-point polymerase chain reaction (PCR) technique with the primer pairs previously
153 described in the literature; Bs-XeF-Bs-XeR, Bs-XvF-Bs-XvR, Bs-XgF-Bs-XgR and Bs-XpF-
154 Bs-XpR (Koenraadt *et al.*, 2009). Twenty-five microliters of the reaction mix were prepared
155 with 2X Reaction Buffer (Thermo Scientific, Cat. No. EP0401), 0.1 mM dNTPs, 0.2 µM
156 forward and reverse primers, 1 U Taq DNA polymerase (Thermo Scientific, Cat. No. EP0401),
157 1 mM Mg+2, 20 ng DNA and nuclease-free water. Thermal cycling was performed on
158 SimpliAmp (Applied Biosystems, USA) instrument under the following conditions: initial
159 denaturation at 95°C for 3 min and followed by 35 cycles of 95°C for 30 s denaturation, 57,4°C
160 for 45 s annealing, 72°C for 1 min elongation. PCR was finalized at 72°C for 10 min a final
161 elongation step. The PCR products were run on 2% agarose gel to control for the presence of
162 amplicons.

163 RST65 and RST69 primer pair (Obradovic *et al.*, 2004) was used to sequence the ATP-
164 dependent RNA helicase (*hrpB*) gene for phylogenetic analysis of the species. The reaction
165 mixes and PCR condition were identical with the molecular diagnosis of pathogenic bacteria
166 strains experiment. PCR products were directly sent to Macrogen Inc. (The Netherl ands) for
167 purification and both-direction Sanger dideoxy sequencing using RST65 and RST69 primers.
168 The DNA sequences were imported to Geneious Prime (20243.0.3) software suit for quality
169 score check, trim, and obtain consensus sequencesfor quality checking, trimming the primer
170 binding sites and obtaining the consensus sequences by assembling both directions reads. A
171 phylogenetic tree was constructed with PAUP 4a using Maximum Parsimony approach
172 (Heuristic search) (Swofford, 2003).

173

174 RESULTS

175 Establishment of a bacterial collection

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

180 **Table 1.** The origin, biochemical, morphological, and pathogenicity traits of isolated *Xanthomonas* strains in this
181 study.

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

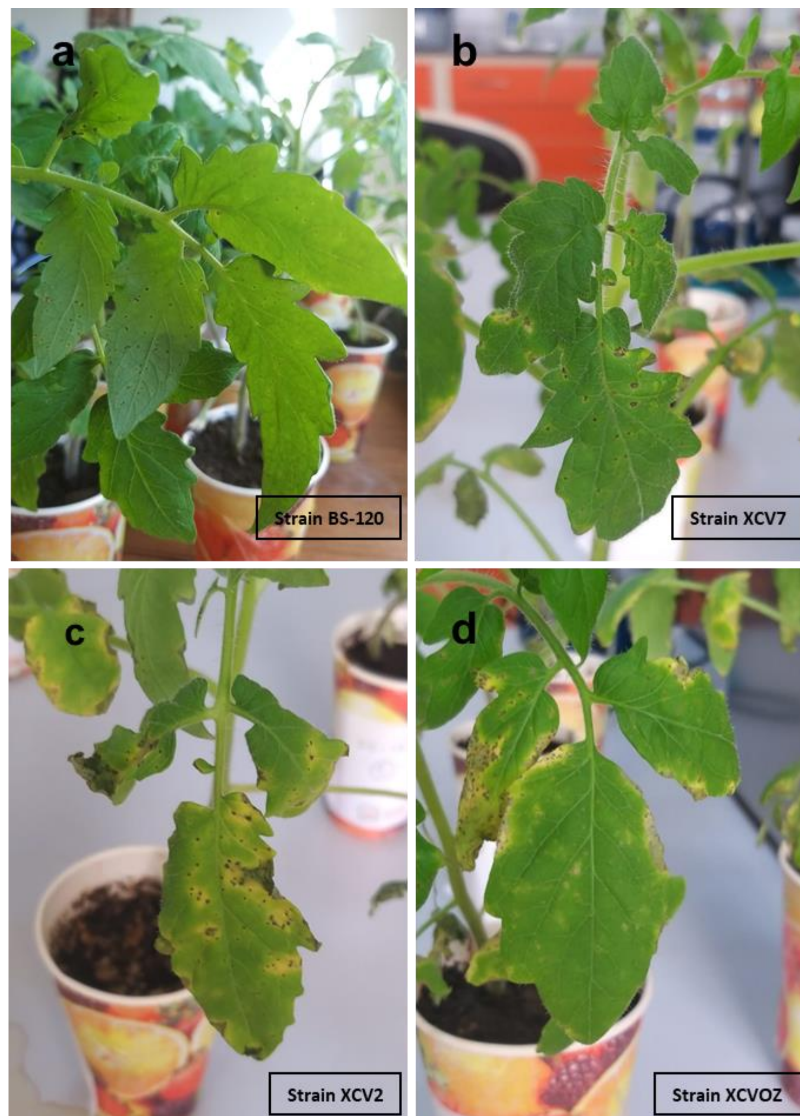
Gr: Gram reaction, Hr: Hypersensitivity (Tobacco) test, Ox: Oxidase test, Ca: Catalase test, Am: Amylolytic activity (K^+-strong positive), Ds: Diseases severity(%), +: Positive, -: Negative

182

183 Phenotypic characterization and pathogenicity of bacterial strains

184 All ten strains isolated from tomato plants triggered a hypersensitive reaction in tobacco plants.
185 These strains formed distinct yellow, circular, mucoid, and shiny colonies when grown on YDC
186 medium. The results of biochemical teste are shown in the **Table 1**. All strains were Gram-
187 negative bacteria with positive catalase activity but lacked oxidase activity. The ability to break
188 down starch (amylolytic activity) varied among the strains. Strains XCV7 showed strong
189 amylolytic activity, whereas XCV1, XCV2, XCV3, XCV5 and XCV7/1 displayed positive
190 result. The remaining strains, XCVO, XCVOZ, XCVOZ1 and XCVOZ2 were negative for

191 amylolytic activity. A gas chromatography system, called the Microbial Identification System,
192 was used to analyze the fatty acid profiles of the ten strains. Based on these profiles, the strains
193 were identified as *Xanthomonas campestris* pv. *vesicatoria* with a similarity index ranging from
194 61 to 78%. The details of the identification and similarity index can be found in the **Table 2**. In
195 addition, these strains were subjected to pathogenicity tests (**Figure 2**). The disease severity
196 values (%) obtained from the pathogenicity tests of the strains are given in the **Table 1**. All ten
197 strains caused disease in cv. 'Süper domates' plants, as shown in **Table 1**. No symptoms
198 appeared on negative control plants. While the reference strain caused severe disease in tomato
199 plants (81% severity), the disease severity caused by the isolated strains ranged from 33.91 to
200 74%. XCV2 was the most virulent strain, causing 74% disease severity in tomatoes.
201



202
203 **Figure 2.** Symptoms induced on tomato plants by inoculations with *X. euvesicatoria* pv. *perforans* (strains XCV2-
204 XCV7), *X. euvesicatoria* pv. *euvesicatoria* strain XCVOZ and *Xanthomonas axonopodis* pv. *vesicatoria* strain
205 BS-120 (control +): (a) Circular water-soaked lesions on the leaves, (b) Initial symptoms of bacterial spot of
206 tomato, (c) Advanced symptoms of bacterial spot of tomato, and (d) Necrotic lesions on the leaves surrounded by
207 chlorotic margins.

208

209 **Molecular diagnosis and phylogenetic analysis of bacterial strains**

210 Since the integrity and the concentrations of DNA extracts (varied between 28.74 ng μL^{-1} to
211 78.16 ng μL^{-1}) were appropriate, we directly proceeded to downstream applications PCR and
212 sequencing. PCR results showed that the Bs-XeF-Bs-XeR primer pair successfully amplified
213 the related DNA region for XCVO, XCVOZ, XCVOZ1 and XCVOZ2 strains whereas the other
214 primers pair did not (**Table 2**).

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

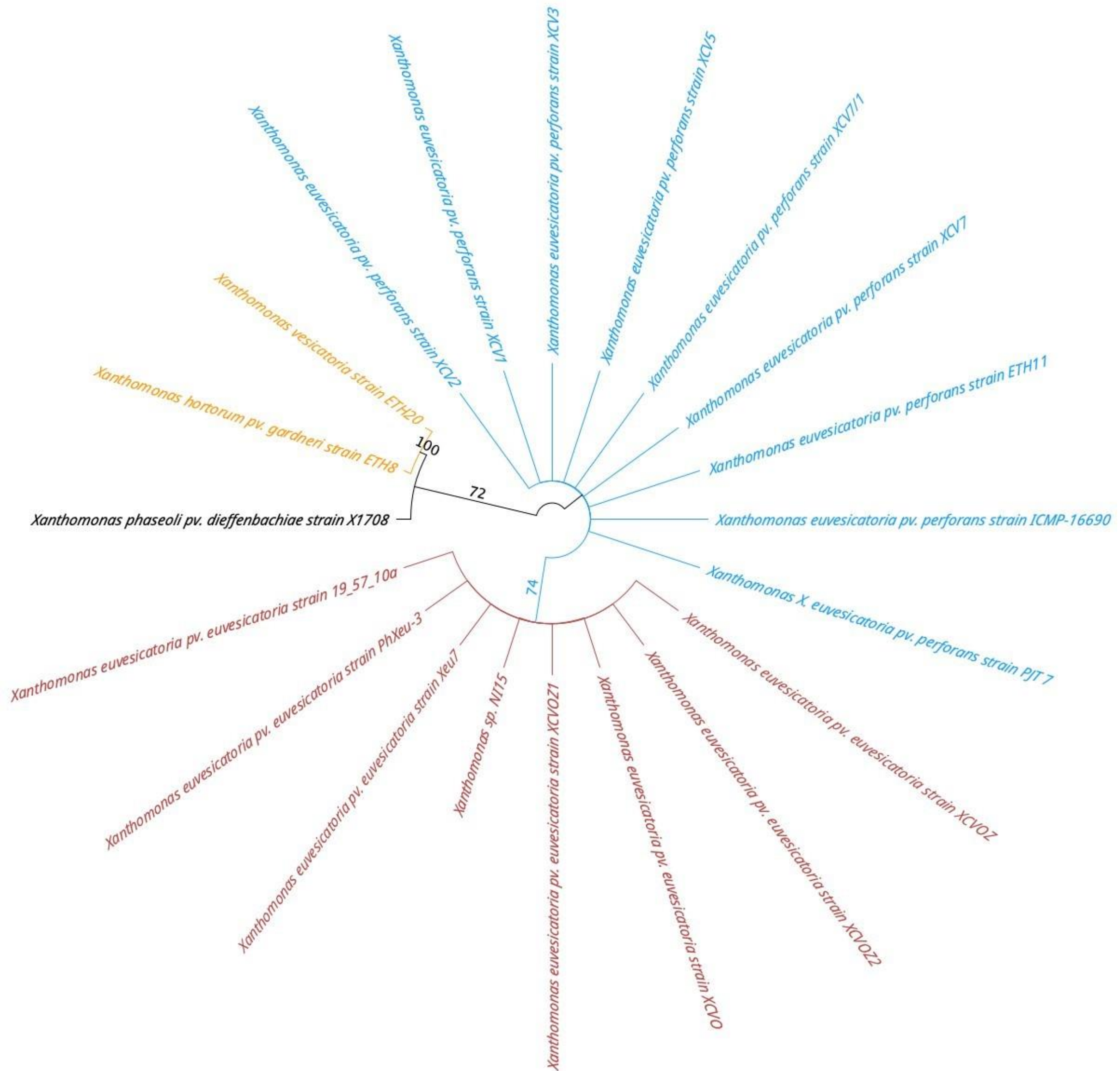
Strains	FAME		Species-specific PCR				Identity based on *hrpB*		
	FSI (%)	**FAME**	**a**	**b**	**c**	**d**	**Species**	**SI (%)**	**Accession***
XCV1	73	*X. campestris* pv. *vesicatoria*	-	-	-	-	*X. euvesicatoria* pv. *perforans*	100	PP505864
XCV2	78	*X. campestris* pv. *vesicatoria*	-	-	-	-	*X. euvesicatoria* 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

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 *NCBI accession numbers of the sequences generated with the present study. +: Positive, -: Negative.

217

218 The RST65 and RST69 primer pairs were used to amplify the *HrpB* gene region for sequencing.
219 Sanger dideoxy sequencing was successful for all samples. The assembled and trimmed
220 sequence quality scores varied between 90.7% to 98.7% which is reliable for further
221 phylogenetic analysis. Additionally, all newly generated sequences were deposited in GenBank
222 (Table 2). We performed BLASTn search for for each sample and obtained the best hit
223 results.The alignment length of the total 20 samples was 237 base pair (bp).
224 The cladogram consisted of three main clades (orange, blue and violet) and an outgroup (**Figure**
225 **3**). *X. phaseoli* pv. *dieffenbachiae* strain X1708 was placed as an outgroup to *X. euvesicatoria*
226 pv. *perforans* and *X. euvesicatoria* pv. *euvesicatoria* samples as supposed. *Xanthomonas*
227 *hortorum* pv. *gardneri* and *X. vesicatoria* were placed in first clade (violet), *X. euvesicatoria*
228 pv. *perforans* strains placed in the second (blue) clade and *X. euvesicatoria* pv. *euvesicatoria*

229 strains placed in the third clade (orange). The violet clade placed as sister group to the orange
230 and blue clades.



231

232 **Figure 3.** Phylogenetic relationships among 20 strains of the *Xanthomonas* species complex were obtained with
233 sequence analysis using *HrpB* gene region by Maximum Parsimony approach. The bootstrap (1000 replicates)
234 consensus values were placed on the branches.

235

236 **DISCUSSION**

237 Various bacterial pathogens can infect tomato plants at different stages of growth, causing a
238 range of diseases (Lin and Wang, 2010). This study focused on the presence of bacterial spot
239 disease in tomato plants from the Iğdır plain. Ten bacterial strains were obtained from these
240 tomato plants, and all strains exhibited varying degrees of virulence (Table 1), causing bacterial
241 spot symptoms on tomato plants. *Xanthomonas* species possess various virulence factors,
242 including adhesins for plant attachment, flagella and fimbriae for motility and adhesion, and

243 exopolysaccharides and lipopolysaccharides for interaction with the plant environment.
244 Additionally, secretion systems inject effector molecules that manipulate plant processes, while
245 extracellular degrading enzymes break down plant cell walls for nutrient access. This
246 coordinated deployment of virulence factors empowers *Xanthomonas* to overcome plant
247 resistance and establish disease (An *et al.,* 2020). While these virulence factors play a crucial
248 role in *Xanthomonas* species colonization and infection of tomatoes, further investigation is
249 needed to pinpoint additional critical factors specific to our strains. Tomato bacterial spot is
250 caused by a group of related bacteria within the *Xanthomonas* genus (Jones *et al.*, 2004). To
251 identify and differentiate among these specific bacteria, researchers now rely on a combination
252 of molecular and biochemical techniques (Araújo *et al.*, 2012). While conventional methods,
253 such as biochemical tests have been used to differentiate between *Xanthomonas* species (Jones
254 *et al.*, 1998), the emergence of new, unique strains can challenge this approach and lead to
255 misidentification.
256 FAME analysis can be used as a preliminary screening tool to differentiate between broad
257 groups of bacteria based on their overall fatty acid makeup (Gilbride, 2014; Kunitsky *et al.*,
258 2006). FAME analysis was used to characterize the fatty acid profiles of the bacterial strains in
259 this study. All bacterial strains were identified as *X. campestris* pv. *vesicatoria* by FAME with
260 a similarity index ranging from 0.55-0.78. In the 1990s, researchers identified two distinct
261 groups within *X. campestris* pv. *vesicatoria*: group A and group B (Stall *et al.*, 1994; Vauterin
262 *et al.*, 1990). Group A strains were uniformly negative for amylolytic and pectolytic activity,
263 while group B strains were strongly active in both (Bouzar *et al.*, 1994). Our study found similar
264 variation. Six strains displayed amylolytic activity, which is consistent with group B and *X.*
265 *euvesicatoria* pv. *perforans* strains (Jones *et al.*, 2004). However, the remaining four strains did
266 not exhibit amylolytic activity, consistent with group A and *X. euvesicatoria* pv. *euvesicatoria*.
267 While FAME analysis and biochemical tests provided valuable insights, these findings suggest
268 they may not be sufficient for definitively identifying all four tomato-associated *Xanthomonas*
269 pathogens. However, FAME analysis shows promise as a rapid pre-screening tool to identify
270 potentially pathogenic *Xanthomonas* strains in tomatoes. For conclusive identification,
271 complementary techniques might still be necessary.
272 A more reliable approach for diagnosing *Xanthomonas*-caused plant diseases utilizes species-
273 specific primers (Pan *et al.*, 1999; Suk Park *et al.*, 2006). These primers target specific DNA
274 sequences that are unique to each *Xanthomonas* species, allowing for more accurate
275 identification. Previously, methods like the RST 65/69 primers (Leite *et al.*, 1995) were used,

276 but they amplified DNA from a broader group of *Xanthomonas* species. This broader targeting
277 could lead to misidentification, as these primers produced the same sized amplicon not only for
278 tomato bacterial spot strains but also for other unrelated *Xanthomonas* species. Researchers
279 have developed numerous sets of primers specifically designed to identify different
280 *Xanthomonas* species that cause bacterial spot disease (Araújo *et al.*, 2012; Astua-Monge *et al.*,
281 2000; Cuppels *et al.*, 2006; Koenraadt *et al.*, 2009; Moretti *et al.*, 2009). Species–specific
282 primer pairs (BS-XeF/BS-XeR, BS-XvF/BS-XvR, BS-XgF/BS-XgR and BS-XpF/BS-XpR)
283 designed by Koenraadt *et al.* (2009) were used to differentiate these tomato-associated
284 *Xanthomonas* species in this study. PCR using the BS-XeF/BS-XeR primer set identified the
285 expected 173-bp amplicon specific for *X. euvesicatoria* pv. *euvesicatoria* in only four strains.
286 None of the remaining strains produced amplicons when using this primer set. None of the
287 strains were amplified with any other species-specific primer sets tested. PCR using the BS-
288 XeF/BS-XeR primer set and the subsequent sequencing of the *hrpB* gene yielded concordant
289 results. Four out of ten tomato strains were identified as *X. euvesicatoria* pv. *euvesicatoria* using
290 both methods, while the remaining six strains clustered with *X. euvesicatoria* pv. *perforans*
291 **(Figure 3**).
292 However, *X. euvesicatoria* pv. *perforans* strains in this study were not detected using the
293 species-specific primer pair Bs-XpF/Bs-XpR. (Osdaghi *et al.*, 2017) reported similar findings
294 with this primer set. The limited detection of *X. euvesicatoria* pv. *perforans* in this study using
295 the Bs-XpF/Bs-XpR primers aligns with observations by Osdaghi *et al.* (2017) who suggested
296 that these primers might not be sensitive enough to capture the full global diversity of *X.*
297 *euvesicatoria* pv. *perforans*. This is further supported by the fact that all tested strains originated
298 from a single location near the Iranian border in Iğdır, Türkiye. A wider geographical range of
299 strains might be necessary to comprehensively evaluate the effectiveness of these primers for
300 *X. euvesicatoria* pv. *perforans* detection.
301 Despite extensive research on pathogenic *Xanthomonas* in tomatoes in Türkiye, researchers
302 haven't discriminated against exact species using molecular techniques for the current four
303 species (Aysan and Sahin, 2003; Basim *et al.*, 2004; Mirik and Aysan, 2009). A study
304 performed by Eryigit (2016) in Türkiye were tested ten *Xanthomonas* strains. Eight of these
305 strains were identified as *X. euvesicatroia* using both biochemical tests and species-specific
306 PCR analyses. The remaining two strains were identified as *X. euvesicatoria* pv. *perforans*
307 solely through biochemical testing. According to recent whole-genome sequencing study, two
308 bacterial spot pathogens have been reported in Türkiye: *X. euvesicatoria* pv. *euvesicatoria* on

309 peppers and *X. euvesicatoria* pv. *perforans* on tomatoes, peppers, and eggplants (Subedi *et al.*,
310 2023). This study confirms the findings of previous studies on bacterial spot pathogens.
311 Although bacterial spot disease was reported in Iğdır (Sunyar *et al.*, 2021), this is the first study
312 to identify *X. euvesicatoria* pv. *euvesicatoria* and *X. euvesicatoria* pv. *perforans* as the specific
313 bacteria causing the disease in tomatoes grown on the Iğdır Plain.
314
315 **CONCLUSIONS**

316 This study investigated the causal agents of bacterial spot symptoms on tomatoes cultivated in
317 the Iğdır plain (Türkiye). Ten *Xanthomonas* strains, isolated from these symptomatic plants,
318 underwent pathogenicity testing and were subsequently identified using molecular and
319 biochemical techniques. As a result, it was determined that the *X. euvesicatoria* pv.
320 *euvesicatoria* and *X. euvesicatoria* pv. *perforans* strains are the causal agents of bacterial spot
321 disease in tomatoes grown in the Iğdır plain.
322
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