

## IDENTIFICATION OF CAUSAL DISEASE AGENT(S) OF CHERRY BACTERIAL CANCKER IN MARMARA REGION OF TURKEY

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### Abstract

Bacterial cancer diseases of cherry (*Prunus avium* L) caused by *Pseudomonas syringae* (*P. syringae*) is one of significant diseases in fruit producing areas of the World and can cause major economics losses. So, the survey study was conducted to obtain the *P. syringae* pathovar(s) in Marmara Region of Turkey. Tress with symptoms of bacterial cancker were found in six cities of nine cities visited and ninety-five of hundred-seven orchards between 2016 to 2018 years in Marmara Region. Gram negative bacterial isolates were isolated from margin of necrotic tissue. All isolates were based on pathogenicity, LOPAT, and GATTa tests, 63 isolates were identified as *P. s. pv. syringae* (*Pss*). For molecular study, showed that all isolates produced on approximately 752 bp DNA fragment in PCR performed with primer set *syrB1-syrB2*. However, the results of MALDI- TOF MS analysis, the isolates were identified as *Pss*. According to the obtained results, it was concluded that *Pss* cause necrosis of cherry trees and its prevalence rate was determined as 58.88% in Marmara Region of Turkey.

Keywords: Bacterial cancker, cherry, GATTa, MALDI-TOF MS, prevalence rate

### 1. INTRODUCTION

The production of cherries has increased in the last decade in the world. According to data from the Food and Agricultural organization for the 2014-2019 period, the average production of cherries increased from 2.187.770 to 2.595.812 tons. In Turkey, the average production of cherries is around 6 million tons in 2019. So, Turkey takes first place in producing cherries in the World (FAO, 2019) However, the bacterial cancker has long been a problem in the production of sweet cherries worldwide. Bacterial cancker is caused by *P. syringae* *pv. syringae* (*Pss*) and *morsprunorum* (*Psm*), and is an important disease of sweet cherry. Disease symptoms include blossom blast and spur dieback, leaf and fruit lesions, canckers with associated gummosis of woody tissue, loss of scaffold limbs, and overall decreased fruit yields (Kennelly et al., 2007; Spott et al., 2010).

Both phenotypic (including pathogenicity tests) and genetic methods are used for *P. syringae* identification. The pathogenicity test, in many cases even avirulent isolates can be allocated to the correct pathovar using the appropriate identification test (Young et al., 1992). Multiple tests on many different hosts are necessary to accurately determine the pathogenicity and host range of a *P. syringae* isolate (Gilbert et al., 2009). The different pathovars and races of *P. syringae* isolate from cherry have been distinguished and characterized by physiological and biochemical tests (Burkowicz & Rudolph, 1994; Garrett et al., 1966; Luz, 1997; Vicente et al., 2004) including the

LOPAT (levan production, oxidase activity, pectolytic activity, arginine dihydrolase, hypersensitive reaction on tobacco) and GATTa tests (gelatin liquefaction (G), aesculin hydrolysis (A), tyrosinase activity (T) and tartrate utilization (Ta). (Lelliott et al., 1966; Latorre & Jones, 1979; Vicente et al., 2004). Especially, GATTa tests are specifically useful for discrimination *Pss* and *Psm* (Latorre and Jones, 1979). However, The phytotoxin coronatine produced by *Psm* isolates and syringomycin produced by *Pss*. So, PCR assay is used to identify by primers based on coronatine and syringomycin gene (Bereswill et al., 1994; Sorensen et al., 1998; Bultreys and Gheysen, 1999; Bultreys et al., 2006).

This study aimed to determine the causal agent(s) in the Marmara region of Turkey and characterized by biochemical, pathogenicity, molecular tests, and MALDI-TOF MS.

## 2. MATERIALS AND METHODS

### Survey

In 2016-2018 period, the survey studies were conducted in Balıkesir, Bursa, Canakkale, Edirne, Kırklareli, Kocaeli, Istanbul, Tekirdag and Yalova in Marmara region of Turkey. The samples were collected from infected sweet cherry trees.

### Isolation and Pathogenicity

The tissue pieces from infected plant samples were surface-sterilized for 15 sec in 70% ethanol and macerated in 1 ml of phosphate buffer. The isolation was carried out of King's B agar (King et al., 1956). The plates were incubated for 2-3 days at 25 °C.

Pathogenicity of the obtained isolates was tested by inoculation of bacterial suspension ( $10^8$  cfu/ml) on immature sweet cherry fruits and bean pods using a medicinal syringe (Klement, 1990; Gavrilovic, 2006). The steril distilled water (SDW) and Naip-1 (*Pss*) isolate were used as a control in the study.

### Biochemical and Physiological Characterization

The tests were carried out a formation of levan and activity of oxidase, pectolytic enzymes, arginine dihydrolase, and hypersensitive reaction on tobacco leaves (LOPAT tests), as well as gelatin and aesculin hydrolysis, tyrosinase production, and use of tartaric acid (GATTa tests) (Lelliott et al., 1996; Kaluzna et al., 2010).

### MALDI-TOF Mass Spectrometer

This method is based on revealing of microorganisms protein profile with ionization of protein structure and these ionized mass pass through the electrical field. Profiles that were obtained from microorganisms compare with a database of system thus identification is made by this way. Ribosomal proteins are used in identification which is less affected by environmental conditions. For bacterial isolates identification, the raw spectra of the unknown bacteria were used for pattern matching against the reference spectra of the database. The results of the pattern-matching process were expressed as proposed by the manufacturer, with log(scores) values ranging from 0 (no similarity) to 3 (absolute identity) (Carolis et al., 2012; Ziegler et al., 2012; Uysal et al., 2019).

### Genetic analysis of *P. syringae* pathovar

Total genomic DNA was prepared by De Boer and Ward (1995). Determination of the presence of genes coding for synthesis of the bacterial phytotoxins syringomycin and coronatine was carried out by PCR with *syrB* encoding synthesis of syringomycin with primers *syrB1* (5'-CTTCCGTGGTCTTGATGA-3') and *syrB2* (2 (5'TCGATTTTGCCGTGATGAGT-3'), and *cfl* encoding coronatine synthesis with primers *CFLF* (5'-GGCGCTCCCTCGCACTT-3') and *CFLR* (5'-GGTATTGGCGGGGGTGC-3'), amplifying a 752 bp band for *syrB* and 650 bp band *cfl*

were expected. The PCR amplification procedure used is described by Bereswill et al. (1994) and Abbasi et al. (2013). The PCR products were electrophoresed on 1% TBE agarose gel.

### 3. RESULTS AND DISCUSSIONS

#### Survey

The pathogen is known as a major problem in fruit-producing areas worldwide (Kennelly et al., 2007). Recently the cases of bacterial canker increased and pathogen became more prevalent (Spott et al., 2010). According to the study, a total of one hundred seven cherry orchards were surveyed in the Marmara region of Turkey. In Edirne, Kocaeli, and Yalova were not obtained infected plant samples. Also, our results indicated the prevalence of bacterial canker disease is 58.88% in the Marmara region of Turkey (Table 1).

*Table 1. Survey study in Marmara region of Turkey*

Location	Number of isolates			Number of orchards
	2016	2017	2018	
Balıkesir	-	5	-	11
Bursa	-	3	5	8
Canakkale	2	6	4	12
Edirne	-	-	-	3
Kırklareli	-	4	-	5
Kocaeli	-	-	-	2
Istanbul	-	-	2	5
Tekirdag	2	17	13	54
Yalova	-	-	-	3

#### Isolation and Pathogenicity Test

After isolation, the bacterial colonies that were white and fluorescent on King's B medium were selected and purified. A total of eighty-two bacterial isolates were obtained from sweet cherry orchards. 63 isolates were pathogenic to both sweet cherry fruits and bean pods inducing dark brown and sunken lesions of variable size 7- 10 days after inoculation (Moragrega et al., 2003). Reference *Pss* Naip1, used as a positive control in the pathogenicity test, also caused the same symptoms, while no symptoms were observed on tissue inoculated with SDW (Table 2). Balaz et al. (2016) indicated that *Pss* isolates from the sweet cherry in Serbia were showed the same pathogenicity result on immature cherry fruits. The symptoms were described that large, dark, and sunken spots (Gasic et al., 2012; Bulbul et al., 2015; Balaz et al., 2016).

#### Biochemical and Physiological Characterization

Sixty-three isolates were Gram(-) and produced fluorescent pigment on King's B medium like reported by Lelliott and Stead (1987). All isolates produced levan on SNA (Sucrose Nutrient Agar) medium and also water-soaked and necrotic lesions on tobacco leaves. Therefore, they belonged to the *Pseudomonas* group 1a. The GATTa tests are known differentiation within the *P. syringae* species. *Pss* isolates are hydrolyzed gelatin and aesculin (Schaad et al., 2001; Gilbert et al., 2009). The results of GATTa tests, sixty-three isolates showed the ability to gelatine liquefaction and aesculin hydrolysis. So, all isolates were identified as *Pss* (Table 2).

A previous study indicated that biochemical tests, though useful for the identification of bacterial genera and species, are often not discriminatory enough to determine *P. syringae* pathovars (Morris et al. 2000).

### MALDI-TOF Mass Spectrometer

The log(score) values of isolates were measured higher than 2.0. Also, the isolates were showed pattern-matching with reference in the database as *Pss*.

### Genetic analysis of *P. syringae* pathovar

Pathovars of *P. syringae*, associated with stone fruits, produced several well-characterized phytotoxic compounds that can be used for pathovar differentiation. Like syringomycin is the main virulence factor for *Pss*. The syringomycin is developed necrotic tissues on cherry. So, *Pss* is distinguished from *Psm*. Detection of the *syrB* gene was routinely used for the determination of *Pss* (Gilbert et al., 2009; Kaluzna et al., 2010; Popovic et al., 2021). Detection of *syrB* gene, PCR amplification with primer *syrB1* and *syrB2* was produced 752 bp band on agarose gel-like as reference *Pss* isolate Naip1. However, PCR amplification with primer CFLF and CFLR was not observed 650 bp band size. So, the isolates were identified as *Pss* (Table 2).

Table 2. Determination of *P. syringae* isolates from cherry

Isolate	Locality	Years of isolation	Gram reaction	LOPAT	GATTa	Pathogenicity	PCR		Result
							<i>syrB</i>	<i>cfl</i>	
TK1	Tekirdag	2018	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK2		2018	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK3		2018	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK4		2018	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK5		2018	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK6		2018	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK7		2017	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK8		2017	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK12		2017	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK13		2017	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK17		2017	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK18		2017	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK19		2017	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK20		2017	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK21		2017	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK24		2017	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK25		2017	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK26		2017	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK27		2017	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK28		2017	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK29		2017	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK30		2017	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK31		2017	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK33		2018	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK34		2018	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK36		2018	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK37		2018	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK38		2018	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK40		2016	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK41		2016	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK42		2018	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>
TK44		2018	Gram(-)	+++++	+++	+	+	-	<i>Pss</i>

BaK2	Balıkesir	2017	Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
BaK3		2017	Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
BaK4		2017	Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
BaK7		2017	Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
BaK8		2017	Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
BuK1	Bursa	2017	Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
BuK2		2017	Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
BuK3		2017	Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
BuK6		2018	Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
BuK7		2018	Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
BuK8		2018	Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
BuK9		2018	Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
BuK10		2018	Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
ÇK1		Çanakkale	2016	Gram(-)	+----+	+++	+	+	-	<i>Pss</i>
ÇK2			2016	Gram(-)	+----+	+++	+	+	-	<i>Pss</i>
ÇK4	2017		Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
ÇK5	2017		Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
ÇK6	2017		Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
ÇK7	2017		Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
ÇK8	2017		Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
ÇK9	2017		Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
ÇK10	2018		Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
ÇK11	2018		Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
ÇK12	2018		Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
ÇK13	2018		Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
KK1	Kırklareli		2017	Gram(-)	+----+	+++	+	+	-	<i>Pss</i>
KK2		2017	Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
KK3		2017	Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
KK4		2017	Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
IsK1	İstanbul	2018	Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
IsK2		2018	Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	
Naip1 (ref. <i>Pss</i> )	Tekirdağ	2014	Gram(-)	+----+	+++	+	+	-	<i>Pss</i>	

#### 4. CONCLUSIONS

In the survey, studies were conducted nine different cities in Marmara region of Turkey. However, six cities out of nine were obtained the bacterial canker. The result of this study indicated that bacterial canker is present and spread in cherry orchards in the Marmara region of Turkey. Characterization of the isolates indicated that *Pss* is caused by bacterial canker in cherry orchards in the Marmara region. All tests were supported the *Pss*. it was concluded that *Pss* cause necrosis of cherry trees and its prevalence rate was determined as 58.88% in Marmara Region of Turkey.

#### 5. ACKNOWLEDGEMENTS

This study was a part of the Ph.D. thesis namely Identification, genotypic characterization of causal agent of cherry bacterial canker in marmara region, and determination of susceptibility against different compound.

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