

## Identification of *Pseudomonas syringae* isolates causing bacterial blight symptoms on pear and quince trees in Yozgat province of Turkey

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**Abstract.** *Pseudomonas syringae* is a polyphagous phytopathogenic bacterial disease agent associated with more than 180 plants species. The bacterial agent causes economically important diseases on several species of fruit trees in Turkey. In 2019, pear and quince trees in several orchards in districts of Yozgat province located in the Central Anatolian Region of Turkey showed bacterial blight symptoms that differed from symptoms of fire blight caused by *Erwinia amylovora*. Bacterial isolates were obtained from necrotic pear and quince tissue on King's medium B. Occurrence of pure greyish colonies producing green-blue fluorescent pigment was checked after incubation at 26 °C for 48 h. All purified isolates (n = 5) were Gram-negative, aerobic, and negative for oxidase, induced hypersensitive reaction on tobacco leaves, did not cause soft rot on potato slices, and formed levan-positive colonies on Nutrient Sucrose Agar medium. The identification of bacterial isolates was further confirmed by MALDI-TOF MS and molecular analysis. Identification of these isolates was confirmed by matching with reference *Pseudomonas syringae* pv. *syringae* DSM6693 type strain by using MALDI-TOF MS analysis. For molecular identification was performed by sequencing the *rpoD* gene that was amplified with primer pair PsrpoD-FNP1/Psrp-Dnprpcr1. Obtained partial sequences of *rpoD* gene of five representative isolates showed 100% nucleotide similarity with *rpoD* gene sequences of available *P. syringae* pv. *syringae* isolates from the GenBank. Pathogenicity test revealed that these isolates caused typical disease symptoms on the inoculated pear and quince parts. Re-isolation of the pathogen was obtained from the margins of necrotic lesions on inoculated plant parts. Based on the expressed symptoms, biochemical characteristics, pathogenicity and molecular analysis, the causal disease agent was identified as *Pseudomonas syringae* pv. *syringae*.

**Keywords:** Bacterial blight, *Pseudomonas syringae* pv. *syringae*, pear, quince, *rpoD*

### Introduction

The *Pseudomonas syringae* complex includes many different taxonomical related species which comprises isolates in different biochemical, immunological, and molecular characteristics (Ivanović et al., 2017; Gutiérrez-Barranquero et al., 2019). Among more than sixty pathovars in the *P. syringae* complex, *P. syrin-*

*gae* pv. *syringae* van Hall is the most polyphagous bacterium that can cause disease on nearly 180 plant species of different cultivars around the world (Kerko-ud et al., 2002; Kennelly et al., 2007; Lamichhane et al., 2014; Gomila et al., 2017; Gašić et al., 2018).

Bacterium infects wide range of fruit trees such as apricot, cherry, citrus, hazelnut, pear, peach and plum, as well as other woody plant species resulting in eco-

nomic crop losses (Canfield et al., 1986; Roos & Hattingh, 1987; Akbaba & Ozaktan, 2021; Oksel et al., 2022). The control of the pathogen is difficult as it has a broad host range and represent a big concern because causing great damages on young and older trees. Infection of woody parts is also crucial on fruit trees because cankers can girdle branches which could eventually kill trees (Yildiz et al., 2016).

The disease agent was found responsible for bacterial blossom blast and dieback of young pear shoot tips of pear trees (McKeen, 1955; Jones & Aldwinckle, 1990). The bacterium was isolated from infected blossom buds of symptomatic pear trees with blighting and blasting of floral structures in Spain (Montesinos & Vilardell, 1989). On fruit trees, *P. syringae* pv. *syringae* mostly infects stone fruits (Kotan & Sahin, 2002; Akköprü, 2016; Yıldiz et al., 2016; Soylu et al., 2020a; Ertimurtas & Özaktan, 2020; Oksel et al., 2022) and is capable of causing disease on hazelnut (Karahana et al., 2016), citrus (Mirik et al., 2005) including pome fruits in different regions of Turkey (Kotan et al., 2006). Until Kotan et al. (2006) reported that *P. syringae* pv. *syringae* is the second abundant bacterial species in pear and quince grown in the Eastern Anatolia Region of Turkey, it was not associated with infections on pome fruits. *P. syringae* pv. *syringae* was already detected in apricot plants in different locations in the same region (Kotan & Sahin, 2002).

The aim of this study was to identify the bacterial isolates obtained from diseased pear and quince plants in Yozgat province by biochemical, pathogenicity and molecular tests.

## Material and Methods

During spring of 2019, leaves, twigs and shoots of pear and quince plants showing blight symptoms with necrotic lesions were collected from different locations of Yozgat province located in the Central Anatolia Region of Turkey. Blossom blast, dried with necrosis, twig dieback, bark necrosis and trunk canker symptoms were observed that differ from the symptoms of fire blight caused by *Erwinia amylovora*. Bacterial isolation was performed using internal fragments of infected tissue. Samples were surface sterilized in 1% NaOCl for 3 min, and rinsed three times in sterile water. After tissue homogenization in saline buffer

(0.85% NaCl) the suspension was left for bacterial releasing for 45 min. The extract was streaked onto King's B medium (KB) plates and left for incubation at 28 °C for 72 h. Cultures of isolates (n = 5) whitish-grey in colour with slightly lobed margins were purified and stored at -20 °C in 30% glycerol in Luria-Bertani broth for identifications tests (Kotan et al., 2006).

Potassium hydroxide (KOH) for determination of Gram reaction (Suslow et al., 1982), catalase activity, oxidative/fermentative metabolism of glucose (Hugh & Leifson, 1953) and LOPAT (*L*: levan production; *O*: oxidase production; *P*: pectinolytic activity; *A*: arginine dihydrolase production; and *T*: tobacco hypersensitivity) tests were applied for isolates from quince (n = 3) and pear (n = 2) (Lelliot & Stead, 1987; Schaad et al., 2001).

Ethanol-formic acid-acetonitrile protein extraction was applied for the bacterial mass of the pure cultures grown for 24–36 h on Tryptic Soy Agar (TSA, Merck, Darmstadt, Germany) medium (Soylu et al., 2021). One µl of the extract was dropped onto the MALDI target, dried and overlaid with 1 µl of acyano-4 hydroxy-cinnamic acid matrix solution. Data was analysed with BIOTYPER™ 1.1 software (Bruker Daltonics GmbH, Bremen, Germany) (Duman & Soylu, 2019).

The presence of the *syrB* gene encoding syringomycin production was determined by PCR amplification using primer pairs B1 (5'-CTTCCGTGGTCTTGATGAGG-3') and B2 (5'-TCGATTTTGCCGTGATGAGTC-3') (Sorensen et al., 1998). For further identification, *rpoD* gene (sigma subunit of RNA polymerase) was amplified using primer pair P<sub>syrpD</sub>-FNP1 (5'-TGAAGGCGARATCGAAATCGCCAA-3') and P<sub>syrpD</sub>-Dnprpcr1 (5'-YGCMGWCAGCTTYTG-CTGGCA-3') (Parkinson et al., 2011). In each PCR amplification with individual primer pair, suspected colony was picked up with a sterile pipette tip and directly placed into the PCR reaction tube prepared in 25 µl final volume including 12.5 µl of 2× master mix (MyTaq Mix, Bioline, England), 1 µl of forward primer, 1 µl of reverse primer, 1 µl of DMSO and 9.5 µl of sterile water. Amplifications were performed with a thermal cycler (Bio-Rad, T100) and conditions were set up for a touch-down PCR as follows: 4 min at 95 °C, 10 cycles with 30 s at 94 °C, 30 s at 62–53 °C (decrease 1 °C per cycle), 1 min at 72 °C and 5 min at 72 °C, in addition 24 cycles at a constant 53 °C using

the same parameters (Aksoy et al., 2017). The PCR products were separated on 1.5% agarose gel in  $1 \times$  TAE buffer, stained with ethidium bromide, and visualized using UV transilluminator. DNA molecular weight marker (Hyperladder, 1 kb, Bioline, England) was used for estimation of the size of PCR products.

Consensus sequences for *rpoD* gene were generated using Chromas pro (version 1.7.6). Nucleotide sequences were searched for BLASTn analysis in NCBI GenBank. Multiple alignments were applied for *rpoD* gene sequences using ClustalW in MEGA 6 with reference sequences of different *P. syringae* pathovars retrieved from the NCBI GenBank (Tamura et al., 2013). Jukes and Cantor model was used for inferring evolutionary distances (Jukes & Cantor, 1969).

Pathogenicity test was performed on the leaves of 2-year-old pear and quince saplings which was inoculated with bacterial suspensions ( $10^8$  cfu ml<sup>-1</sup>) of isolates by injecting into leaves. The second test was applied on the stem of young twigs detached with sterilized scalpel by placing 50  $\mu$ l of bacterial suspension ( $10^8$  cfu ml<sup>-1</sup>) into the wounds, and then covered with parafilm. Sterilized water was used as a negative control (Koh et al., 2012; Araujo et al., 2020).

## Results and Discussion

In 2019, bacterial blight symptoms were observed on pear and quince plants (Fig. 1a and 1b). Pure whitish-grey colonies were obtained on KB plates that produced blue-green fluorescence under UV light. Biochemical test of five isolates (Pss1, Pss2, Pss3 from quince; and Pss4, Pss5 from pear) revealed that all were Gram-negative, showed aerobic metabolism of glucose, negative for oxidase, induced hypersensitive reaction on tobacco plants, did not cause soft rot on potato slices and formed levan-positive colonies on nutrient sucrose agar (NSA) medium. According to these characteristics all isolates were classified into LOPAT Ia group which includes *P. syringae* pathovars, as stated by Ivanović et al. (2017). In several studies, MALDI-TOF MS has been found to be quite accurate for plant pathogenic fungal and bacterial species identification than conventional diagnostic methods (Panda et al., 2013; Singhal et al., 2015; Uysal et al., 2019; Aktan & Soylu, 2020; Soylu et al., 2020b). For faster and reliable taxonomical identification of representative five isolates, MALDI-TOF MS analysis was applied for their pure cultures. Isolates was determined as *P.*

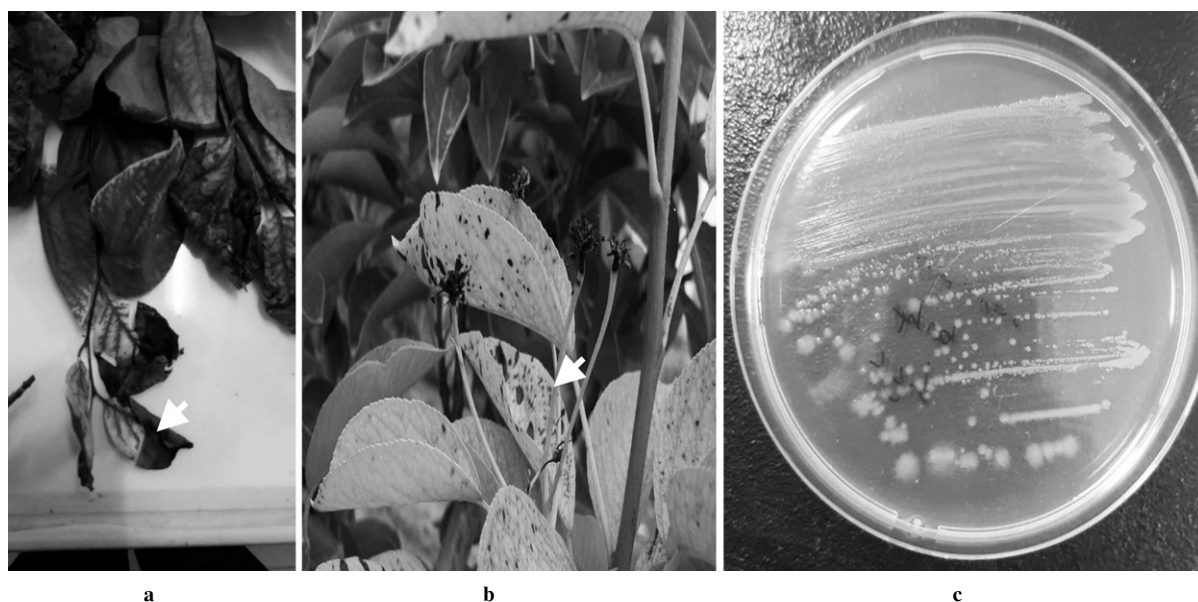


Fig. 1. a) Infected quince samples showing blight symptoms on leaf (indicated by arrow), b) Diseased pear tree with blossom blight with necrotic black tissue (indicated by arrow), c) Purified bacterial culture of *P. syringae* pv. *syringae* isolate Pss1  
Sl. 1. Zaraženi uzorci dunje sa simptomima na listu (označeno strelicom), b) Zaraženo stablo kruške u fazi cvetanja sa nekrotičnim crnim tkivom (označeno strelicom), c) Čista bakterijska kultura *P. syringae* pv. *syringae* izolat Pss1.

*syringae* pv. *syringae* which matched with the protein profiling of reference strain *P. syringae* pv. *syringae* DSM 6693. The score values ranged from 2.022 to 2.105 that exceeded the threshold of 2 value indicating secure identity. In many cases, MALDI-TOF MS based identifications have shown resolution and reproducibility which is better than gel-based protein or DNA finger printing techniques (Saleeb et al., 2011; Singhal et al., 2015; Kurt et al., 2017).

Further, tested isolates were identified as *P. syringae* pv. *syringae* using molecular identification techniques. Toxin-based detection methods have been useful in identifying *P. syringae* pv. *syringae* isolates (Sorensen et al., 1998). In PCR with primer pairs B1 and B2, a 752-bp PCR fragment was obtained for *sybB* gene which is a significant virulence factor for determining a cyclic lipodepsinonapeptide syringomycin. *rpoD* gene is a suitable biomarker for phylogenetic and evolutionary relationship investigation of species of *Pseudomonas* spp. (Sarris et al., 2012). Amplified

PCR products of *rpoD* gene (700 bp) were sequenced and obtained partial nucleotide (nt) sequences (~666 to 682 nt) of isolates showed 100% similarity with *rpoD* gene sequences of *P. syringae* pv. *syringae* isolates (accession numbers: MK791201, KC852117, KC852111, KC852108) available in the NCBI GenBank (Mulet et al., 2008; Mulet et al., 2010). Phylogenetic tree based on the *rpoD* gene sequences revealed grouping of isolates from this study in the same clade together with reference *P. syringae* pv. *syringae* isolates (accession numbers: MK791201, KC852117, KC852111, KC852108) (Fig. 2).

Pathogenicity of five isolates was confirmed on stems of 2-year-old pear and quince saplings. Typical necrotic lesions appeared on the test plants in the both tests. Leaf necrosis on the place of bacterial suspension inoculation was observed after 5–7 days. After two weeks twigs on inoculated plants showed leaf yellowing that later turn brown to black with necrosis on the woody sections. The pathogenic isolates were re-

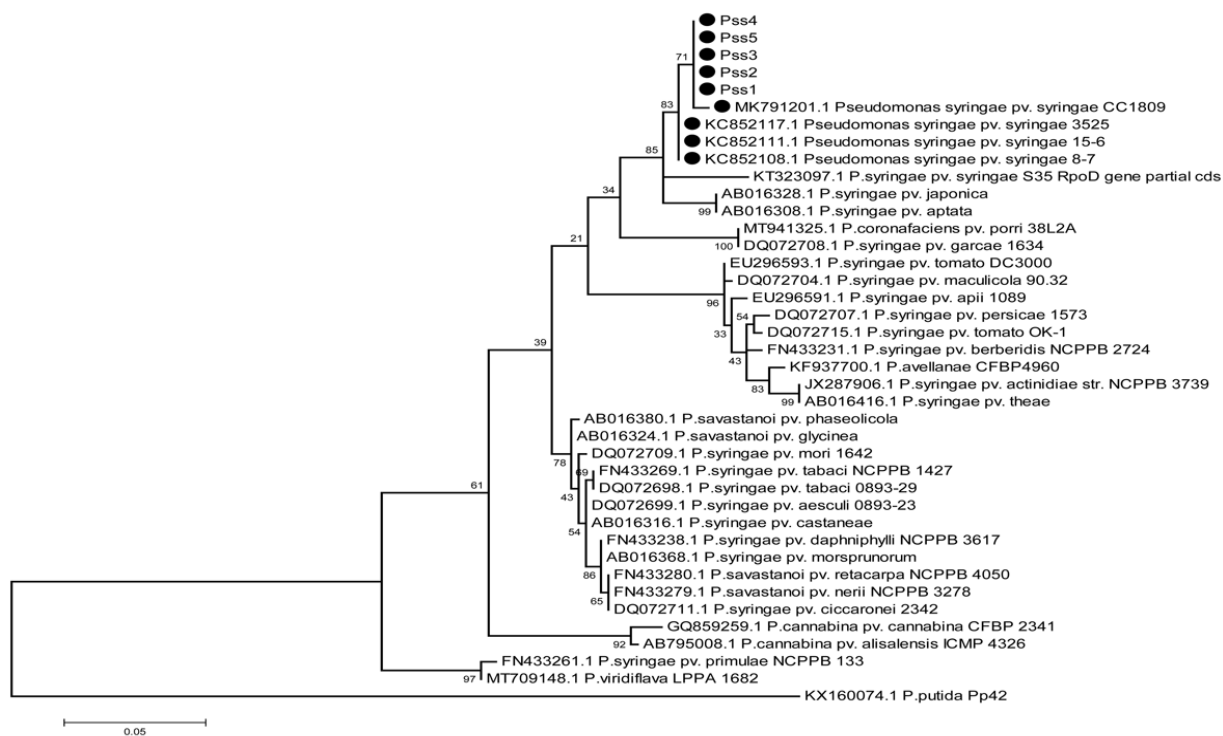


Fig. 2. Phylogenetic tree inferred with 40 *rpoD* sequences of *Pseudomonas* species. NCBI accession numbers of the respective *Pseudomonas* species are given in the tree. *P. putida* was used as outgroup.

Sl. 2. Filogenetsko stablo rekonstruisano od *rpoD* sekvenci 40 *Pseudomonas* vrsta. NCBI pristupni brojevi odgovarajućih *Pseudomonas* vrsta su dati u stablu. *P. putida* je korišćen kao outgroup.

isolated from lesions on inoculated leaves. The results of our research on the occurrence and identification of isolates obtained from pear and quince plants in Yozgat province are in line with previous reports on *P. syringae* pv. *syringae* infection of apple (Lee et al., 2015; Araujo et al., 2020), pear and quince (Panagopoulos & Crosse, 1963; Kotan et al., 2006; Mansvelt & Hattingh, 2007).

## Conclusion

This paper reports the first presence of bacterial blight disease on pear and quince plants caused by *P. syringae* pv. *syringae* in Yozgat province. The identification of pathogenic isolates was confirmed by combining biochemical, pathogenicity, MALDI-TOF MS and PCR based molecular techniques. Certain precautions methods need to be taken by the producers to prevent the spread of the pathogen in the region.

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**IDENTIFIKACIJA IZOLATA *Pseudomonas syringae* UZROČNIKA SIMPTOMA BAKTERIOZNE PLAMENJAČE NA STABLIMA KRUŠKE I DUNJE U PROVINCIJI YOZGAT U TURSKOJ****Murat Öztürk<sup>1\*</sup>, Soner Soylu<sup>2</sup>**<sup>1</sup>Univerzitet Yozgat Bozok, Poljoprivredni fakultet, Departman za zaštitu bilja, Yozgat, Turska

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*Pseudomonas syringae* predstavlja polifagnu, fitopatogenu bakteriju koja se povezuje sa simptomima bolesti prisutnih kod više od 180 biljnih vrsta. Uzročnik je ekonomski značajnih bolesti različitih vrsta voćaka u Turskoj. Tokom 2019. godine, na stablima kruške i dunje u brojnim voćnjacima provincije Yozgat, smeštene u centralnom delu turskog regiona Anatodlia, uočeni su simptomi bakteriозne plamenjače koji su se razlikovali od simptoma tipičnih za *Erwinia amylovora*. Bakterijski izolati su dobijeni iz nekrotičnog tkiva kruške i dunje na King B podlozi. Pojava čistih sivkastih kolonija koje proizvode zeleno-plavi fluorescentni pigment proverena je nakon inkubacije na 26 °C u trajanju od 48 h. Svih pet prečišćenih izolata su bili gram-negativni, aerobni i negativni na oksidazu. Izazivali su hipersenzitivnu reakciju na listovima duvana, ali nisu izazivali meku trulež na kriškama krompira. Formirali su levan pozitivne kolonije na hranljivim podlogama sa saharozom i agarom. Identifikacija bakterijskih izolata je dalje potvrđena MALDI-TOF ma-

senom spektrometrijom (MS) i molekularnom analizom. Identifikacija ovih izolata potvrđena je upoređivanjem sa dostupnim *Pseudomonas syringae* pv. *syringae* DSM6693 sojem korišćenjem MALDI-TOF MS analiza. Molekularna analiza je sprovedena putem sekvenciranja *rpoD* gena koji je amplifikovan sa parom prajmera PsrpoD-FNP1/Psrp-Dnprpcr1. Dobijene parcijalne sekvence *rpoD* gena pet proučavanih izolata pokazale su 100% nukleotidnu identičnost sa sekvencama *rpoD* gena izolata *P. syringae* pv. *syringae* dostupnog u banci gena. Testom patogenosti potvrđeno je da ovi izolati izazivaju tipične simptome na inokulisanim delovima kruške i dunje. Reizolacija patogena je dobijena sa ivica nekrotičnih lezija na inokulisanim delovima biljke. Na osnovu ispoljenih simptoma, biohemijskih karakteristika, patogenosti i molekularnih analiza, patogen je identifikovan kao *Pseudomonas syringae* pv. *syringae*.

**Ključne reči:** bakteriозna plamenjača, *Pseudomonas syringae* pv. *syringae*, kruška, dunja, *rpoD*