

Occurrence of *Pseudomonas syringae* pv. *syringae* the causal agent of bacterial canker of stone fruits in Guilan province of Iran

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Abstract

Pseudomonas syringae pv. *syringae* causes canker, leafspots and necrosis of the bark of cherry, plum, and peach fruit trees. Symptoms caused by this pathogen on leaves, blossoms, and fruit, reported as common else where, are rare in Guilan cherry orchards. In this research, during survey from cherry, plum, and peach orchards in different areas of Guilan province (Talesh, Hashtpar, Astaneh-Ashrafieh and Lahijan), samples were taken from infected tissues of disease trees. For isolation of bacteria causing disease, infected tissue were crushed in bacteriological saline (0.85% w/v NaCl) and 100 μ L of juice was cultured on nutrient agar (NA) and King's B medium. Strains of bacteria rod-shaped, gram negative and aerobic bacterium were isolated. The strains produced Levan on media including sucrose. All strains made Hypersensitive Reaction (HR) on tobacco and geranium leaves. All of the isolated bacteria were oxidase, nitrate, tween 80 hydrolysis, indole and starch hydrolysis negative and could not rot potato tuber slices, produced H₂S, and grew at 36°C. The isolates could use citrate and urease. The isolates produced acid from sorbitol, galactose, myo-inositol, manitol, xylose, maltose and sucrose. Their gelatin test were positive. Based on morphological, physiological, biochemical, pathogenicity properties and total cellular protein profiles (SDS-PAGE), the predominate pathogenic type was identified as *P. s.* pv. *syringae*. This is the first report of the existence of *P. s.* pv. *syringae* on stone fruit trees in Iran.

Key words: Stone fruit trees, *Pseudomonas syringae* pv. *syringae*, canker, Iran

Introduction

Bacterial canker disease is caused by *Pseudomonas syringae* pv. *syringae*, a bacterium. Although most serious on sweet cherries, bacterial canker also affects peaches, prunes, plums, apricots and almonds. Trees already weakened by frost, wounds, early pruning, water stress and poor nutrition are vulnerable to cankers (Anonymous, 2004; Ogawa *et al.*, 1995). Disease outbreaks are sporadic and more frequent on sweet cherry than on sour cherry. *P. s.* pv. *syringae* is found on peach (Jones and Sutton, 2004). Bacterial canker is an important disease affecting stone fruits in all production areas (Roberts and Smith, 2002). The disease attacks most parts of the tree. Leaves on the terminal portions of cankered limbs and branches may wilt and die in summer or early autumn if girdled by a canker. Occasionally, large scaffold limbs are killed. Leaf and fruit infection occur sporadically, but they can be of economic significance in years with prolonged wet, cold weather during or shortly after bloom (Jones and Sutton, 2004). The pathogen produces a potent phytotoxin called syringomycin that is estimated to nearly double its virulence (Gross *et al.*, 1997). The bacteria can survive from one season to the next in bark tissue at canker margins, in apparently healthy buds and systemically in the vascular system (Jones and Sutton, 2004). Cankers can continue to develop in lateral branches and the trunk. This disease has been difficult to study and control, as the pathogen is very widespread, lives as an epiphyte on the host and weeds, invades host tissues without inducing symptoms, and causes disease that has symptoms similar to those caused by other pathogens (Roberts and Smith, 2002). Cherry trees are commonly hosts of the causal organism, but disease does not occur unless the climate

is conducive and the host is predisposed (Timothy and Kupferman, 2003). Young cherry trees are the most seriously affected, as trunks are often girdled or severely damaged. Trees affected during the first three seasons after planting are often killed outright or grow so poorly that they must be removed (Jones and Sutton, 2004). Molecular techniques (AFLP) are used for distinguishing between both pathovars, as well as for the assessment of the genetic diversity of the pathogen. In view of relating this diversity to differences in pathogenicity, a method for artificial infection was developed (King *et al.*, 1954). We studied the presence of *P. s.* pv. *syringae* on stone fruit trees such as cherry, plum, and peach orchards in the Guilan province of Iran.

Materials and methods

Bacterial isolations: Samples were collected from orchards in Talesh, Hashtpar, Astaneh-Ashrafieh and Lahijan during 2002–2003. Small tissue pieces from stem lesion margins, surfaces of cankers and leaf tissue showing necrotic lesions and blight symptoms were removed aseptically, ground in bacteriological saline (0.85% w/v NaCl), and left at room temperature (20°C) for 10 min. The suspensions were streaked onto Nutrient Agar (NA) and King's medium B (KB) and incubated at 26°C. Bacterial colonies growing from the suspensions were re-streaked onto KB to obtain single colonies. Isolates were routinely grown on KB at 26°C and stored at 4°C for up to 2 weeks. For long-term storage bacterial strains were stored in freezing medium at -80°C.

Biochemical and physiological tests: Strains were characterized based on the following tests: Gram test in 3% KOH (Sulsow

et al., 1982), oxidative/fermentative test (Hugh and Leifson, 1953) production of fluorescent pigment on KB, hypersensitive reaction (HR) in tobacco and geranium leaves (Lelliot *et al.*, 1987), oxidase test, levan formation, catalase, urease, gelatin liquefaction, litmus milk, salt tolerance (5%) and gas formation from glucose. In addition, tests for arginine dehydrolase, hydrogen sulfide production from peptone, reducing substance from sucrose, tyrosinase casein hydrolase, nitrate reduction, indole production, 2-keto gluconate oxidation lecithinase, starch hydrolysis, phenylalanine deaminase, esculin and Tween 80 hydrolysis and optimal growth temperature were performed as per Schaad *et al.* (2001). The presence of DNase was tested on DNA agar (Diagnostique Pasteur, France). Carbohydrate utilization using Ayer basal medium was carried out and the results were recorded daily upto 8 days (Hildebrand, 1988).

Pathogenicity test on leaves, fruit and branches of cherry: Characteristic colonies of *P. s. pv. syringae* that grew on NA and KB were subcultured. Pathogenicity tests were carried out on leaves, immature sweet cherry fruit and branches of young shoots of cherry. Leaves were removed from young shoots and sterilized with 70% ethanol. In toward of midrib was injured T shape and then 50 μ L droplet of bacterial suspension (3×10^8 CFU mL⁻¹) was placed on them. The leaves were maintained under humid (95%) conditions at 27°C for 10 days. Immature sweet cherry fruit assay was carried out essentially as described by Jones (1971) and Latorre and Jones (1979). Immature sweet cherry fruits were collected during September. The fruits were surface sterilized with 70% ethanol and 100 μ L of bacterial suspension (3×10^8 CFU mL⁻¹) was injected on them.

Branches of young cherry shoots were placed in an Erlenmeyer flask with water. Shoots, 45 cm long, were tip-inoculated by injection of 100 μ L of bacterial suspension 2×10^8 CFU mL⁻¹ with a hypodermic needle; they were maintained at 26°C for 6 weeks. For each strain tested, five branches were used. Control were treated with sterile distilled water.

SDS-PAGE: Electrophoresis of soluble proteins was carried out in a discontinuous SDS polyacrylamide gel according to the method of Laemmli (1970), with some modifications as described by Rahimian (Rahimian, 1995). Strains were grown on NA medium at 24 °C for two days. A bacterial suspension in distilled water was prepared in Eppendorf tube and centrifuged at 13,000 x g for 12 min. The pellet was washed and resuspended in sterile distilled water. The cell suspension optical density was adjusted to 1.0 at 630 nm. Each sample was mixed with 5x sample buffer [sample buffer: 63 ml, Tris-HCl (pH 6.8), 10 % (v/v) glycerol, 2.0% (w/v) SDS and 0.25% (w/v) bromophenol blue] and heated at 95°C for 5 min. To each 950 μ L sample buffer, 50 μ L of 2-mercaptoethanol, as a reducing agent, was added just before boiling. Samples were centrifuged at 15,000 x g for 10 min. Fifty μ L of soluble proteins was loaded in each well in a 13 x 17 cm polyacrylamide slab with 0.75 mm thickness. Proteins were fractionated in 10% resolving gel at a constant current of 20 mAmps for four hrs. The gel was stained in methanol, water and acetic acid (5: 5: 1) containing 0.5% coomassie brilliant blue G250 overnight and destained in the same solution without dye. The gel was kept in 7% acetic acid.

Antibiotic sensitivity test: *P. s. pv. syringae* strains were tested for antibiotic sensitivity using antibiogram disks. A bacterial

suspension was spread on nutrient agar medium. Two disks for each antibiotic were placed on medium. After 48 hrs of incubation at 28°C, inhibition zone was measured.

Results and discussion

Biochemical and physiological tests: All strains were gram, oxidase, catalase negative, and unable to utilize glucose under anaerobic conditions (Table 1). None of the strains were able to produce reducing compounds from sucrose or show lecithinase, arginine dihydrolase activity or produce gas from glucose. All strains were esculin positive and capable of hydrolyzing gelatin, None of the strains were able to hydrolyze Tween 80, produce indole, reduce nitrate and oxidize 2-keto-gluconate. All strains of *P. s. pv. syringae* were able to produce syringomycin and showed ice nucleation activity. All strains were able to utilize citrate, L-Lysine and produce acid from manitol, xylose, D (+) galactose, inositol, maltose, sorbitol, manose and sucrose. None of the strains were capable of utilizing L-arabinose, trihalose and L-tartrate. The presence of DNase was tested on DNA agar (Diagnostique Pasteur, France).

Pathogenicity test: All strains caused dark brownish spots with depressions on the surface of immature cherry fruits 7-10 days after inoculation, whereas a slight discoloration was observed in the control. Gumming occurred at the margins of the cankers and often was heavy. The strains also caused water-soaked spots and necrosis in the injured region on cherry leaves after a week. These symptoms did not occur in the control. Further, inoculation of young shoots on cherry trees with individual strains caused chlorotic spots that eventually became necrotic and dried. Cankers typically ooze amber-colored gum and often become entry sites for borers (Richard, 2002). These symptoms did not occur in the control.

Serious infections have occurred on young trees that were wetted by rain or irrigation within a few days of planting or after suckers were removed from trunks. Frost, especially when closely followed by rain or heavy dew, leads to bacterial blast of blossoms. In areas of the world that have cool, wet winters, infection of pruning wounds during the fall or winter is common (Roberts and Smith, 2002; Beers *et al.*, 1993).

Protein profile: Total protein pattern of isolates were compared to standard strain. Protein bands of strains were nearly similar to protein bands of standard strain of *P. s. pv. syringae* (Fig. 1). Analysis of the ERIC fingerprints from *P. s. pv. syringae* strains showed that the strains isolated from stone fruits formed a distinct cluster separate from most of the strains isolated from other hosts. These results provide evidence of host specialization within the diverse pathovar *P. s. pv. syringae* (Little *et al.*, 1998).

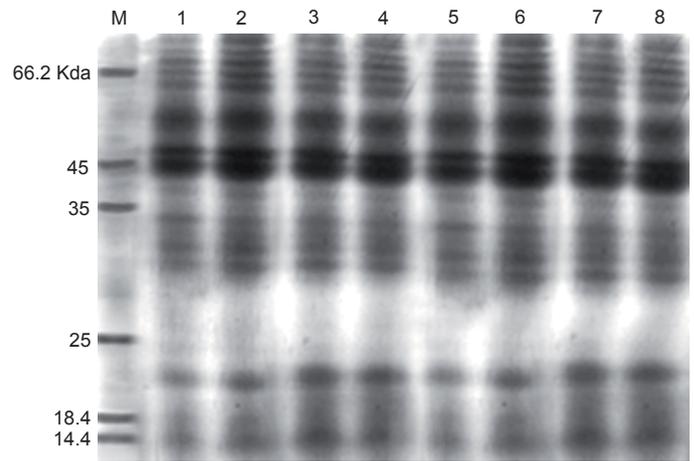
Antibiogram test: *P. s. pv. syringae* strains were tested in duplicates for sensitivity toward 16 different antibiotics (Table 2). They were classified into three groups: resistant (without an inhibition zone), relatively sensitive (inhibition zone with a diameter less than 10 mm) and sensitive (inhibition zone greater than 10 mm in diameter).

Control of bacterial canker is often difficult and sometimes impossible. As with most plant problems, keep trees as healthy as possible so that they are less vulnerable to attack. Most chemical

Table 1. Phenotypic characteristics of *P. syringae* pv. *syringae* strains tested

Characteristics	Isolates of <i>P. s. pv. syringae</i>
Gram reaction	-
Oxidative/Fermentative	-
Fluorescent pigment	+
HR on tobacco	+
Ice nucleation	+
Growth at 39°C	-
Syringomycin production	+
Leaf blight on pear	+
Pectinase	-
Acetoin	-
Arginine dihydrolase	-
Levan formation	+
Nitrate reduction	-
Catalase	-
Tween 80 hydrolysis	-
Oxidase	-
Starch hydrolysis	-
Gelatin hydrolysis	+
Esculin hydrolysis	+
DNase activity	+
Indole formation	-
H ₂ S from cysteine	-
Casein hydrolysis	-
Urease	+
MR	-
Utilization of	
L-lysine	+
Citrate	+
lecithinase	-
Growth in 5% NaCl	-
Acid from	
L-Arabinose	-
Myo-Inositol	+
Manitol	+
Xylose	+
Trihalose	-
Maltose	+
L-tartrate	-
D-Galactose	+
D-Sorbitol	+
Sucrose	+
D-Rafinose	-
D-Manose	+
D-Glucose	+
Cellobiose	-
Inolin	-
Fructose	+
Lactose	-
Ribose	-
D-Adnitol	-
Glycerol	+

control of this disease is based on fungicides containing copper (Roberts and Smith, 2002). The strategy of planting clonal cherry material, supposedly resistant to bacterial canker, can become very risky because of the high phenotypic and genetic variation of *P. syringae* isolated from wild cherry trees, which was demonstrated by the diagnostic techniques (De Cuyper and Steenackers, 2003).

Fig. 1. SDS-PAGE analysis of total soluble proteins of bacterial isolates from stone fruit trees, M, Marquer, lane 1, *Pseudomonas syringae* pv. *syringae* CFBP 2212 (standard strain), lanes 2 to 8 isolates of *P. s. pv. syringae* in polyacrylamide gelTable 2. Antibiotic sensitivity (25 µg/disk) of *P. syringae* pv. *syringae* strains used in this study

Antibiotic	Sensitive (%)	Relatively Sensitivity (%)	Resistant (%)	Range of inhibition zone (mm)
Oleardoomycin	6	6	88	11.5
Phosphomycin	0	0	100	0
Spectinomycin	0	26	74	4
Streptomycin	6	33	61	4.75
Doxycycline	13	40	47	4.1
Cephalexin	13	27	60	6.6
Novobiocine	13	27	60	5.3
Ampicillin	0	0	100	0
Rifamycin	46	64	0	10.3
Kanamycin	6	60	34	10.6
Oxytetracycline	73	27	0	4.3
Chloramphenicol	100	0	0	4

The bacteria overwinter inside the host plants, usually along the edge of cankers that grew the previous season, or in infected but symptomless buds or other host tissue (Roberts and Smith, 2002). Bacteria spread from overwintering sites to grow epiphytically on tree, leaf, flower and weed leaf surfaces. Moist, cool weather favors the spread and growth of bacterial colonies. Rain and wind serve as the primary means of local dispersal. Temperatures below 80 °F, dry weather, and low relative humidity cause a rapid decline in epiphytic populations of the bacteria, which survive the summer inside of host tissues. Temperatures over 95°F may greatly reduce the numbers of bacteria surviving inside the plant tissues (Roberts and Smith, 2002).

Isolates of *P. s. pv. syringae* were used in this study, came from various locations within Guilan province and this is first report of *P. s. pv. syringae* revealing high incidence on cherry, plum, and peach fruit trees from Iran. The study indicates the importance of investigation on a large population of isolates in different regions of Iran.

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