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BACTERIAL CANKER CAUSE BY *PSEUDOMONAS SYRINGAE* PV. *SYRINGAE* ON 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 bacterial causal disease, infected tissue were crushed in pepton water then 100µL of juice were cultured on Nutrient Agar (NA) and King's B medium containing Cyclohexamid antibiotic (50 µg mL^{-1}). 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, produce H_2S, and grow in 36°C. The isolates could use citrate and urease. The isolates produce acid from sorbitol, galactose, myo-inositol, manitol, xylose, maltose and sucrose. Their gelatin test were positive. Based on morphological, physiological, biochemical, pathogenicity properties, total cellular protein profiles (SDS-PAGE) and PCR method with specific primers 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.

Keywords 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 (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 a significant disease affecting stone fruits in all world 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 infections 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[3]. 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 (Jones and Sutton, 2004). Cherry trees are commonly hosts of the causal organism, but disease does not occur unless the climate is conducive and the host 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 will be developed. The method of rep-PCR can also assist in the identification of *P. syringae* pv. *syringae* isolates, although it cannot replace inoculation on susceptible hosts such as cherry and lilac(Vicente and Roberts, 2007). We studied the identification 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 isolation

Samples were collected from orchards of pear 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 (King *et al*., 1954) 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 longer-term storage bacterial strains were stored in freezing medium at -80°C.

Pathogenicity test on leaves, fruit and branche 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 µL 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 (Yassad-Carreau *et al*., 1994; Jones, 1971) 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 µL 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 µl of bacterial suspension $2x10^8$ 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.

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 and. Stead, 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 lecitinase, starch hydrolysis, phenylalanine deaminase, esculin and Tween 80 hydrolysis and optimal growth temperature (Schaad et al., 2001). The presence of DNase was tested on DNA agar (Diagonistic Pasteur, France). On Ayer medium were also performed. Carbohydrate utilization using Ayer basal medium was carried out and the results were recorded daily upto 2-8 days (Hildebrand, 1988).

SDS-PAGE

Electrophoresis of soluble proteins was carried out in a discontinuous SDS polyacrylamid gel according to the method of Laemmli (1970) with some modifications as described by Rahimian (1995).

DNA Extraction

For bacterial DNA extraction, the isolates were grown overnight, in nutrient broth (Merck, Darmstadt, Germany), at 26 °C and the DNA was extracted as described by Martins et al., 2005. One tube of 1.5 ml was used to centrifuged the cells at 13,000 x g for 5 min and the pellet was suspended in 200 µl Tris 0.1 mol L-1 and added with 200 µl of lysis solution (NaOH 0.2 N and 1% SDS), mixed and deproteinazed with 700 µl of phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v), homogenized and centrifuged 10 min at 13,000 × g .To precipitate DNA, 700 µl of cold absulate isopropanol was added and spinned, washed in 70% ethanol and centrifuged. Precipitated DNA is dried at room temperature and suspended in 100 µl of water. The method described by Ausubel *et al.* (1996) was performed comparing 30 strains. The samples from the both methods were electrophoresed on 1.5% agarose gels, stained with ethidium bromide and photographed under UV light.

Primers for *P.s.pv. syringae*

The 20–mer oligonucleotid HrpL1, 5'-TTGGCTAGGTATCGCTATGG -'3 and HrpL2 5'-AGGACCCAGTTTTGGAGTGC-'3 were designed and tested for *P.s.pv. syringae* (Alfano *et al.*, 1996).

PCR conditions for amplification and electrophoresis

Amplification was carried out in a 25 µl volume in 0.5 ml microtube using a Hybaid programmable thermal controller. Each 25 µl PCR reaction mixture contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM MgCl2, 200 µM of each nucleotide (dATP,dCTP, dGTP, and dTTP), 0.25 µM of each primer, 100 ng DNA and 1 U of *Taq* DNA polymerase (Promega Corp., Madison, WI). A 25 µl sterile, mineral-oil overlay was added to

reduce evaporation. DNA amplification was carried out in a PTC-100 programmable DNA thermal cycler (MJ Research, Watertown MA). The amplification was performed as follows:, initial 5 min 94°C denaturation; 45 cycles of 1 min 94°C, 1 min 52°C, 1 min 72°C; and 5 min 72°C extension. Amplified fragments were separated in 1.5 % agarose gel using TBE buffer and were visualized and photographed using a Gel Documentation System, GDS 8000 (BioRad., California, USA), after staining with ethidium bromide.

RESULTS AND DISCUSION

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 utiliz L-arabinose, trihalose and L-tartrate. The presence of DNase was tested on DNA agar (Diagonistic 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 occurs at the margins of the cankers and often is heavy (Jones. and Sutton, 2004). 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. 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 (Beers *et al*., 1993; Jones. and Sutton, 2004)

Protein profile

Total protein pattern of isolates 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*.

Detection of *P.s.* pv.*syringae* by direct PCR

All isolates of *P.s.pv. syringae* were identified by specific primers HrpL1 and HrpL2. On agarose gel electrophoresis 1.5%, isolates produced a band 600 bp. and all isolates (expected size). The bands of isolates were similar with isolates standards of CFBP 3077 (Fig. 2). Based on the phenotypic pathogenicity and PCR tests, the causal agent of bacterial Canker on stone fruit was identified *Pseudomonas syringae* pv. *syringae*. The bacteria overwinter inside of 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 above the low 80s □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 of plant tissues. Isolates of. *P. s.* pv. *syringae* were used in this study, came from various locations whitin Guilan province and this is first report of *P. s.* pv. *syringae* at hight incidence on cherry, plum, and peach fruit trees from Iran. It would be to study a large of population of isolates in different regions of Iran.

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

Bacterial tests Charecteristics	Isolates of *P.s.*pv.*syringae*
Gram reaction	-
Oxidative/Fermentative	-
Fluorescent pigment	+
HR on tobacco	+
Ice nucleation	+
Growth at 39°C	-
Syringomycin production	+
Leaf blight on pear	+
Pectinase	-
Acetoin	-
Argenine dihydrolase	-
Levan formation	+
Nitrate reduction	-
Catalase	-
Tween 80 hydrolysis	-
Oxidase	-
Starch hydrolysis	-
Gelatin hydrolysis	+
Esculin hydrolysis	+
DNase activity	+
Indole formation	-
H_2S 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	-
Froctose	+
Lactose	-
Ribose	-
D-Adnitol	-
Glycerol	+

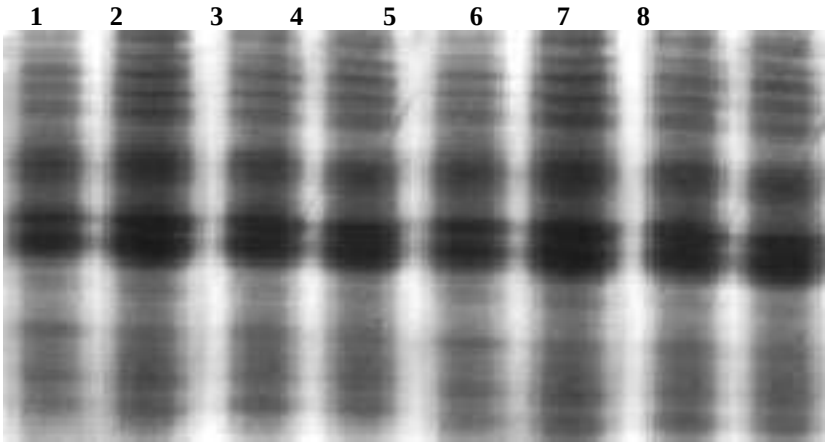

Fig. 1. SDS-PAGE analysis of total soluble proteins of bacterial isolates from ston fruit trees, lane 1, *Pseudomonas syringae* pv. *syringae* CFBP 2212 (standard strain); lanes 2 to 8 isolates of *P. s.* pv.*syringae* from bacterial canker of stone fruits in polyacrylamid gel.

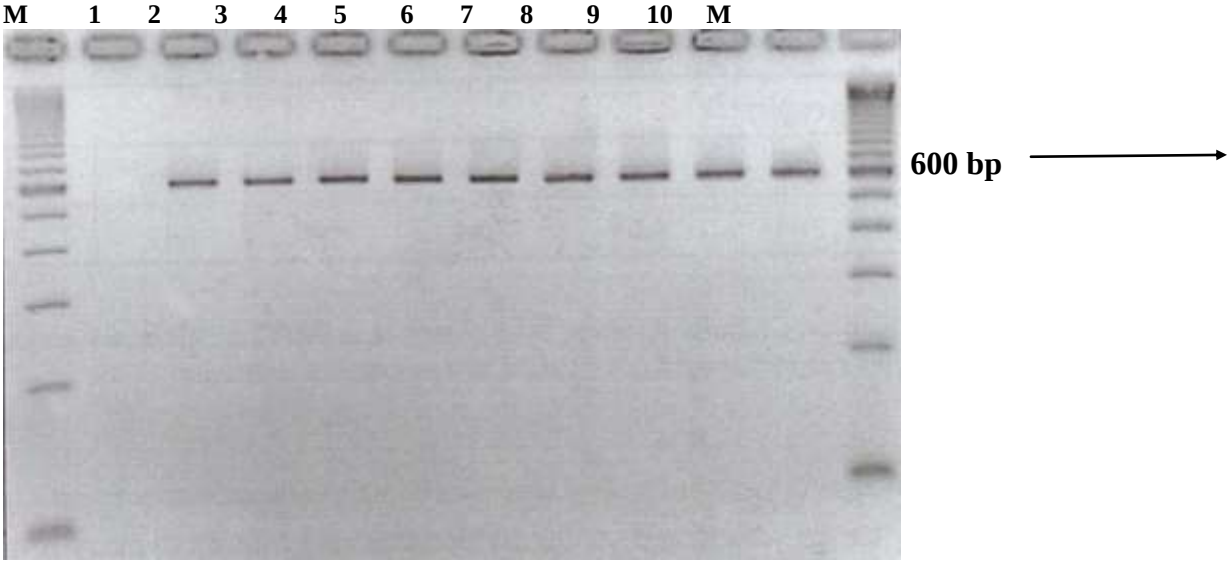

Fig. 2. Agarose gel electrophoresis of products from polymerase chain reaction (PCR) performed on DNA 16S of *Pseudomonas syringae pv syringae* isolates, M, 100 bp DNA marker; lane 1 is negative control (distilled water); lanes 2 to 9, strains of *P.s.* pv. *syringae* isolated from bacterial canker of stone fruits; lanes 10 is posetive control (*P.s.*pv.*syringae* CFBP 3077) showing the amplification the approximately 600 bp.

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