SHORT REPORT
doi: 10.2306/scienceasia1513-1874.2008.34.323

Occurrence of *Pseudomonas syringae* pv. *syringae* the causal agent of bacterial canker on olives (*Olea europaea*) in Iran

Maesomeh Ashorpour[a], Mostafa Niknejad Kazempour[a,*], Mohamad Ramezanie[b]

[a] Department of Plant Pathology, Faculty of Agricultural Sciences, University of Guilan, Rasht P.O. Box. 41635-1314, Iran

[b] Natural Resources of Research, Guilan Province, Iran

* Corresponding author, e-mail: niknejad@guilan.ac.ir

Received 21 Nov 2007
Accepted 24 Jun 2008

ABSTRACT: The cultivation of olive trees is of considerable importance for the olive oil industry in Guilan, Iran. Samples were taken from sunken brown stem lesions on 2-year-old olive trees infected with *Pseudomonas syringae* from various olive groves in Guilan. Based on morphological, physiological, biochemical, pathogenicity properties, and PCR analysis with specific primers, the predominate pathogenic type was identified as *P. syringae* pv. *syringae*. This is the first report of the existence of *P. syringae* pv. *syringae* on olive trees in Iran.

KEYWORDS: olive trees, *Pseudomonas syringae* pv. *syringae*, bacterial canker

INTRODUCTION

The olive (*Olea europaea* L.) is one of the oldest agricultural tree crops and is cultivated for both oil and table olives. Olives are one of the largest crops in the Guilan Province, especially in Manjil and Roudbar, in northern Iran. The olive requires a long, hot growing season to ripen the fruit properly, no late spring frosts that would kill the blossoms, and sufficient winter chill to ensure fruiting[1]. Although the olive tree is affected by some pests and diseases, it has fewer problems than most fruit trees[2]. On olive, bacterial stem cankers and dieback caused by *Pseudomonas syringae* pv. *syringae* are likely to have entered plants through pruning wounds or where freeze/cold injury had caused the stem tissue to crack or peel. Symptoms vary from slow decline of trees and tree death to localized cankers around wound sites[3]. *P. syringae* has been reported on olives from Italy, where it has been found to grow epiphytically on olive leaves[4] and infect freeze-damaged plants[5]. In southern Italy, *P. syringae* was found in association with necrosis and stem girdling at the collar of young olive trees of the Carolea variety and was attributed to a nutritional imbalance which weakens the trees and makes them susceptible to infection[6]. The objectives of the present study were the isolation of the causal agent of bacterial canker on olives in the Guilan province and the identification of the isolates by biochemical, nutritional, pathogenicity, and PCR methods.

MATERIALS AND METHODS

Bacterial isolation

Samples were collected from olive orchards in Roudbar, Aliabad, Manjil, and Loshan 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 by plastic roller in bacteriological saline (0.85% w/v NaCl), and left at room temperature (20 °C) for 10 min. Loopfuls of the bacterial suspension were streaked onto the surface of plates made of Nutrient Agar (NA) and King's medium B (KB) and incubated at 26 °C. In this way, pure bacterial cultures were obtained from the infected tissue. 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 a freezing medium (Luria peptone + glycerol) at –80 °C.

Pathogenicity test on olive stems

Pathogenicity tests[6] were conducted on young potted 18-month-old olive cultivars (Marie, Kroniky, Conservalia, and Arbkin obtained from Spain) using 30 olive bacterial isolates. Stems were wounded with a 25G hypodermic needle. The wound was covered with cotton wool dipped in a suspension of 1×10^7 cfu/ml of each isolate and the inoculation site was wrapped with grafting tape. Control plants were treated with sterile

distilled water. The trees were kept in high humidity (95%) for 24 h before and after inoculation. Following this, the grafting tape and cotton wool were removed, and the olive seedlings were moved outdoors where they were watered overhead twice daily.

Biochemical and physiological tests

Strains were characterized by the Gram test[7] in 3% KOH, the oxidative/fermentative test[8], production of fluorescent pigment on KB, hypersensitive reaction (HR) in tobacco and geranium leaves[9], an 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 sulphide production from peptone, reducing substances 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[10] were performed. The presence of DNAse was tested on DNA agar (Diagonistic Pasteur, France). Carbohydrate utilization was tested for by using a Ayer basal medium and the results were recorded daily for up to 8 days[11]. For each test in this study, a representative isolate has been deposited in the Collection Française de Bactéries Phytopathogèns culture collection. This reference isolate was considered as a typical isolate of *P. syringae* pv. *syringae*.

DNA extraction

For bacterial DNA extraction, 8 isolates were grown overnight in nutrient broth (Merck, Darmstadt, Germany) at 26 °C and the DNA was extracted as described[12]. The cells were pelleted by centrifugation at 13,000 g for 5 min and the pellet was resuspended in 200 µl each of Tris 0.1 mM and lysis solution (NaOH 0.2 N and 1% SDS). The resulting suspension was mixed, deproteinized with 700 µl of phenol/chloroform/isoamyl alcohol (25:24:1 v/v/v), and centrifuged for 10 min at 13,000 g. To precipitate DNA, 700 µl of cold isopropanol was added, centrifuged, washed in 70% ethanol, and centrifuged again. Precipitated DNA was dried at room temperature and was resuspended in 100 µl of water. Electrophoresis was carried out on 1.5 % agarose gels. DNAs were stained with ethidium bromide and were visualized under UV light.

Primers for *Pseudomonas syringae*

Two 20-mer oligonucleotides, i.e. PSF, 5′-TT-GGCTAGGTATCGCTATGG-3′ and PSR 5′-AGG-ACCCAGTTTTGGAGTGC-3′ were designed and synthesized. The primers were used for the amplification of DNA from *P. syringae* pv. *syringae* according to the method described previously[13].

PCR amplification and electrophoresis

PCR amplification was carried out in a 0.5-ml microtube using a Hybaid programmable thermal controller. The reaction (25 µl) contained 10 mM Tris-HCl (pH 9.0), 50 mM KCl, 0.1% Triton X-100, 1.5 mM $MgCl_2$, 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). Steriled mineral oil (25 µl) was overlayed to reduce evaporation. The amplification was performed using the following conditions: initial 5 min at 94 °C, 45 cycles of 1 min at 94 °C, 1 min at 52 °C, 1 min at 72 °C, and 5 min at 72 °C. Amplified fragments were separated in 1.5% agarose gel using TBE buffer and were visualized and photographed using the gel documentation system GDS 8000 (BioRad., California, USA) after staining with ethidium bromide.

RESULTS

Biochemical and physiological tests

All 30 isolates were gram-, oxidase-, and catalase-negative, and were unable to utilize glucose under anaerobic conditions (Table 1). None of the isolates produced reducing compounds from sucrose, or showed lecithinase or arginine dihydrolase activity, or produced gas from glucose. All isolates were esculin positive and were capable of hydrolysing gelatin. None of the isolates were able to hydrolyse Tween 80, produce indole, reduce nitrate, or oxidize 2-keto-gluconate. All isolates of *P. syringae* pv. *syringae* were able to produce syringomycin and showed ice nucleation activity. All isolates were able to utilize citrate and L-lysine and produced acid from manitol, xylose, D(+) galactose, inositol, maltose, sorbitol, manose, and sucrose. None of the isolates were capable of utilizing L-arabinose, trihalose, or L-tartrate. The presence of DNAse was tested on DNA agar (Diagonistic Pasteur, France).

Pathogenicity test

After 5 months, dark sunken lesions were observed at every site inoculated with the olive isolates of *P. syringae*. Subsequently, some of these lesions completely girdled the stems of the trees and death occurred above the inoculation point. The bacteria recovered from these lesions were identified as *P. syringae* pv. *syringae*, confirming Koch's postulates. Cankers typically ooze amber-coloured gum and often

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

Characteristics	I	R
Gram reaction	-	-
Fluorescent pigment	+	+
Levan formation	+	+
Oxidative/Fermentative	-	-
Pectinase	-	-
Arginine dihydrolase	-	-
HR on tobacco	+	+
Ice nucleation	+	+
Growth at 39 °C	-	-
Syringomycin production	+	+
Acetoin	-	-
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	+	+
Trehalose	-	-
Maltose	+	+
L-Tartrate	-	+
D-Galactose	+	+
D-Sorbitol	+	+
Sucrose	+	+
D-Rafinose	-	-
D-Manose	+	+
D-Glucose	+	+
Cellobiose	-	-
Inolin	-	+
Fructose	+	+
Lactose	-	-
Ribose	-	-
D-Adnitol	-	-
Glycerol	+	+

I: Iranian isolates of *P. syringae* pv. *syringae*
R: Reference *P. syringae* pv. *syringae* CFBP 3077

become entry sites for borers[3]. No ooze was observed in these lesions and no fluorescent pseudomonads were recovered. Serious infections occurred on young trees that were wetted by rain or irrigation within a few days of planting or after suckers were removed from the trunks. Every inoculation site developed a superficial raised growth that split the bark. It is most likely that this growth was callus tissue caused by a reaction of the olive to wounding and the presence of

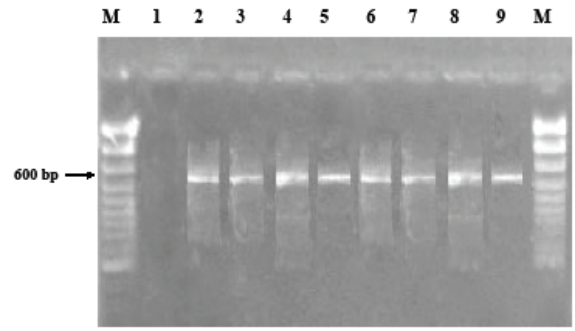

Fig. 1 Agarose gel electrophoresis of products from polymerase chain reaction (PCR) performed on 16S DNA of *P. syringae* pv. *syringae* isolates. M: 100 bp DNA marker; Lane 1: negative control (distilled water); Lane 2: positive control (*P. syringae* pv. *syringae* CFBP 3077) showing the amplification of an approximately 600 bp fragment; Lanes 3–9: strains of *P. syringae* pv. *syringae* isolated from bacterial canker of olive.

P. syringae. The lesions on the artificially inoculated olive plants were not observed for several months after inoculation. This long incubation period may also have occurred in the first year of infection of the olive trees in the orchard.

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

All 8 isolates of *P. syringae* pv. *syringae* were identified by the specific primers PSF and PSR. Electrophoresis of the amplified DNA revealed a 600-bp DNA band of expected size. This band from isolated strains appeared to be similar to that of *P. syringae* pv. *syringae* CFBP 3077 used as standard (Fig. 1). Based on the phenotypic and pathogenicity properties and the results from PCR, the causal agent of bacterial canker on olive was identified as *P. syringae* pv. *syringae*.

DISCUSSION

Isolates of *P. syringae* pv. *syringae* obtained in this study came from various locations within Guilan province and this is first report of *P. syringae* pv. *syringae* at high incidence on olive trees in Iran. All 30 isolates of *P. syringae* pv. *syringae* produced canker on the stem of olive. No significant differences were observed in the degree of disease symptoms. These results suggest that strains isolated from different orchards do not differ in their degree of virulence. Lesions and branch death have been observed in several varieties of olive. Further molecular work is underway to characterize the *P. syringae* pv. *syringae*

isolates from olives, and to test the susceptibility of other varieties.

The infection was more severe in April, with large lesions observed on the main stems and severe branch death occurring on many trees. 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[14,15]. *P. syringae* pv. *syringae* was also recovered from stem lesions on 3-year-old olives cv. Marie in an adjacent planting. The lesions on the artificially inoculated olive plants were not observed for several months after inoculation. This long incubation period may also have occurred in the first year of infection of the olive trees in the orchards[16]. Therefore, the most likely infection period of the olives was in mid-October, where a period of high rainfall coincided with maximum temperatures of 24–28 °C. Certified olive material for export should in any case satisfy the phytosanitary regulations of the importing countries, especially with respect to any of the pathogens covered by the scheme which are also quarantine pests. These bacteria are likely to have entered plants through pruning wounds or where freeze/cold injury had caused stem tissue to crack or peel.

Further research that elucidates the mechanisms eliciting the observed genetic diversity is needed. An understanding of the ecology of natural microbial communities should lead to a more efficient deployment of bacterial populations for disease management. The biological control of bacterial blight on olive by antagonistic isolates in different parts of Iran and the use of resistant cultivars are both topics worthy of further investigation.

REFERENCES

1. Galán C, García-Mozo H, Vázquez L, Ruiz L, Díaz de la Guardia C, Trigo MM (2005) Heat requirement for the onset of the *Olea europaea* L. pollen season in several sites in Andalusia and the effect of the expected future climate change. *Int J Biometeorol* **49**, 184–8.
2. Ferguson L, Sibbett GS, Martin GC (1994) Olive production manual. University of California, Division of Agriculture and Natural Resources: Publication.
3. Hall BH, Cother EJD, Noble D, McMahon R, Wicks TJ (2003) First report of *Pseudomonas syringae* on olives in South Australia. *Australas Plant Path* **32**, 119–20.
4. Ercolani GL (1991) Distribution of epiphytic bacteria on olive leaves and the influence of leaf age and sampling time. *Microbial Ecol* **21**, 35–48.
5. Iannotta N, Lombardo N, Briccoli Bati C, Monardo D (1999) Response of some olive cultivars to minimum late temperatures. *Informatore Agrario* **55**, 59–62.
6. Scortichini M (1997) *Psuedomonas syringae* pv *syringae* associated with decline in olive due to an excess of magnesium. *Informatore Fitopatoloico* **47**, 47–50.
7. Sulsow TV, Schorth MN, Saka M (1982) Application of a rapid method for gram differentiation of plant pathogenic and saprophytic bacteria without staining. *Phytopathol* **72**, 917–8.
8. Hugh R, Leifson E (1953) The taxonomic significance of fermentative versus oxidative metabolism of carbohydrates by various gram negative bacteria. *J Bacteriol* **66**, 24–6.
9. Lelliot RA, Stead DE (1987) Methods for the diagnosis of bacterial disease of plant. Blackwell Scientific Pub., London.
10. Schaad NW, Jones JB, Chun W (2001) Laboratory guide for identification of plant pathogenic bacteria. 3rd eds, APS. St. Paul Minnesota, USA.
11. Hildebrand DC (1998) Pectate and pectin gel for differentiation of *Pseudomonas* sp. and other bacterial plant pathogens. *Phytopathol* **61**, 1430–9.
12. Martins ML, Araújo EF, Mantovani HC, Moraes CA, Vanetti MCD (2005) Detection of the apr gene in proteolytic psychrotrophic bacteria isolated from refrigerated raw milk. *Int J Food Microbiol* **102,** 203–11.
13. Manceau C, Horvais A (1997) Assessment of genetic diversity among strains of *Pseudomonas syringae* by PCR-restriction fragment length polymorphism analysis of rRNA operons with special emphasis on *P. syringae* pv. *tomato*. *Appl Environ Microbiol* **63**, 498–505.
14. Beers EH, Brunner JF, Willett MJ, Warner GM (1993) Orchard pest management. A resource book for the Pacific northwest. Good fruit grower, Yakima, Washington.
15. Jones A.L, Sutton TB (2004) Bacterial Canker, *Pseudomonas syringae* pv. *syringae* and *P. s.* pv. *morsprunorum. Fruit Pathology*, pp 1–2, West Virginia University.
16. Wimalajeewa DLS, Flett JD (1985) A study of populations of *Pseudomonas syringae* pv. *syringae* on stone fruits in Victoria. *Plant Pathol* **34**, 248–54.