SHORT COMMUNICATION

ERWINIA AMYLOVORA CAUSING FIRE BLIGHT OF PEAR IN THE GUILAN PROVINCE OF IRAN

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SUMMARY

Fire blight, caused by *Erwinia amylovora,* is one of the important bacterial diseases of pear trees. It causes blight of different organs of the tree (blossoms, shoots, leaves, fruits, and limbs) and production of exudates. During a survey of pear orchards in different areas of the Iranian province of Guilan (Astaneh, Ashrafieh, Lahijan, and Kiashahr) necrotic shoots and exudates were observed in apple and pear trees. Samples taken from infected tissues were crushed in peptone water and aliquots of 100 µl of the extract were cultured on nutrient agar (NA) and LB containing cycloheximide (50 µg ml^{-1}). A rod-shaped, gram negative, facultative anaerobic bacterium was consistently isolated, which produced levan in sucrose media but not fluorescent pigment in King's B medium. All isolates induced hypersensitive reaction (HR) in tobacco and geranium leaves, were oxidase, nitrate, urease, and indole negative, could not rot potato tuber slices, produced H_2S, and grew at 36°C. The isolates could utilize citrate, arabinose, sorbitol, galactose, and trehalose as carbon source and their gelatin test was positive. Based on morphological, biochemical and physiological characters, and PCR amplification with specific primers, most bacterial isolates were identified as *E. amylovora*. Some isolates from spear orchards were identified as *Pseudomonas syringae* pv. *syringae.* This is the first report of the occurrence of *E. amylovora* on pear trees in the province of Guilan.

Key words: Pear, *Erwinia amylovora*, fire blight, PCR, diagnosis.

Erwinia amylovora (Burrill) is the causal agent of fire blight in most species of the subfamily *Maloideae*, family *Rosaceae*. A *forma specialis* was described from *Rubus* spp. (Starr *et al.,* 1951). *E. amylovora* has a host range of more than 100 species of *Rosaceae.* The disease is believed to be indigenous to North America, from where it has spread worldwide (Vanneste, 1995). Fire blight was first observed in Europe in the UK in 1957 and is now present in 43 countries (Van der Zwet, 2002), including Australia, but not yet in South America nor in most African and Asiatic countries (with the exception of most of those surrounding the Mediterranean sea). *E. pyrifoliae*, a closely related bacterium, was recently described as a new pathogen of Asian pear (*Pyrus pyrifolia*) in Korea, apparently only in the region of Chuncheon (Kim *et al.*, 2001). In Iran *E. amylovora* was first recorded from pear trees in Karaj, then it was found in many orchards in the provinces of Azarbaijan and Ghazvin (Rahnama and Mazarei, 2002).

Plating on semiselective media (Miller and Schroth, 1972; Ishimaru and Klos, 1984) serology (Roberts, 1980; Lin *et al.*, 1987), and molecular assays using colony hybridization (Falkenstein *et al.*, 1988) or PCR (Bereswill *et al.*, 1992; Maes *et al.*, 1996) are currently used for *E. amylovora* identification. Some of these methods were used for ascertaining the presence of *E. amylovora* and *P.s.* pv. *syringae* in pear stands of the Guilan province.

Isolations were made from symptomless flowers and/or infected tissues from orchards in Astaneh Ashrafieh, Lahijan, and Kiashahr collected in 2002-2003. From each orchard, three groups of 25 flowers each were sampled at random. Each sample was suspended in 40 ml of sterile distilled water and serial tenfold dilution were plated on Luria-Bertani (LB) and King's B (KB) media, with 50 µg ml^{-1} of actidione. Plates were incubated for 3 days at 28°C. In orchards with fire blight symptoms, isolations were made also from infected flowers, shoots, and branches with visible cankers. Small pieces of tissue were ground in 5 ml sterile water with a homogenizer (Pro200, Pro Scientific Inc., Monroe, USA) and 100 µl of the homogenate were streaked on LB and on KB plus actidione. At least five samples were tested from each orchard. Liquid cultures for DNA extraction were grown on LB medium. Bacterial isolates were stored at -80 C in 30% glycerol.

Characteristic colonies of *E. amylovora* that grew on LB were subcultured. Pathogenicity tests were carried out on leaves, immature fruits, and young pear shoots.

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Table 1. Phenotypic characteristics of *Erwinia amylovora* and *P.s.* pv. *syringae* strains tested.

	Bacterial tests	
	Isolates of *E. amylovora*	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	-	+
Utilization of:		
L-lysine	-	-
Citrate	+	+
Lecithinase	-	-
Growth in 5% NaCl	+	-
Acid from:		
L-Arabinose	+	-
Myo-Inositol	-	+
Mannitol	-	+
Xylose	-	+
Trihalose	+	-
Maltose	-	+
L-tartrate	-	-
D-Galactose	+	+
D-Sorbitol	+	+
Sucrose	+	+
D-Rafinose	-	-
D-Mannose	-	+
D-Glucose	+	+
Cellobiose	-	-
Inulin	-	-
Fructose	+	+
Lactose	-	-
Ribose	+	-
D-Adonitol	-	-
Glycerol	-	+

Leaves were removed from young shoots and surface sterilized with 70% ethanol, their midrib was wounded and 50 µl droplet of bacterial suspension ($4 \cdot 10^7$ CFU ml^{-1}) was placed on the wound. Leaves were then maintained under humid conditions at 27°C for 10 days (Yassad-Carreau *et al.*, 1994). Immature fruits (cv Khoj) collected in May were inoculated essentially as described by Beer and Rundle (1983). The fruits were surface sterilized with 70% ethanol and cut in transverse slices 1.0 cm thick. For each isolate, three slices were placed on a sterile moist filter paper in a sterile plastic dish and a well (4 mm diameter and 4 mm deep) was cut into the cortical region of each slice using a sterile cork borer. To each well 50 µl of bacterial suspension, at a concentration of 410 CFU ml^{-1}, was added. The slices were maintained under humid conditions at 27°C for 2 weeks.

Branches with young shoots were placed in an Erlenmeyer flask with water. Shoots, 60 cm long, were tip-in-

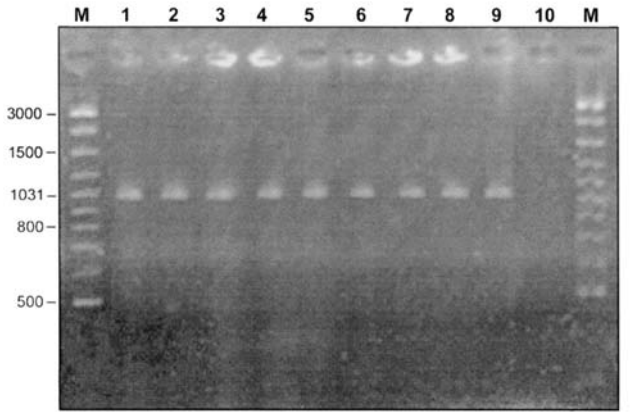

Fig. 1. Agarose gel electrophoresis of products from polymerase chain reaction (PCR) performed on DNA (plasmid pEA29) of *E. amylovora* isolates. M, DNA marker; lane 1: positive control (*E. amylovora* CFBP 1430) showing the amplified product of *ca.* 937 bp; lanes 2 to 9: strains of *E. amylovora* isolated from pear; lane 10: negative control (distilled water).

oculated by injecting 100 µl of bacterial suspension 3·10 CFU ml^{-1} with a hypodermic needle and maintained at 27°C for 4 weeks. For each bacterial strain tested, five branches were used. Controls were inoculated with sterile distilled water.

Strains were characterized based on the following tests: Gram test in 3% KOH (Sulsow *et al.*, 1982), oxidative/fermentative test (Beer and Rundle, 1983), production of fluorescent pigment in KB medium (Hugh and Leifson, 1953), hypersensitive reaction (HR) in tobacco and geranium leaves (Lelliot and Stead, 1987), optimal growth temperature (Schaad *et al.*, 2001) and a series of additional tests as listed in Table 1. The presence of DNase was tested on DNA agar (Diagnostics Pasteur, Marnes-La-Coquette, France). Carbohydrate utilization using Ayer basal medium was carried out and the results were recorded daily up to 3 to 10 days (Hildebrand, 1988).

Bacterial cells grown on LB medium for 24 h were resuspended in sterile distilled water (*ca.* $1{\cdot}10^7$ CFU ml^{-1}), boiled for 10 min, and used for PCR (Manceau and Horvais, 1997) using primers designed from pEA29 plasmid, i.e. Ea1 (5'-CGG TTT TTA ACG CTG GG-3') and Ea2 (5'-GGG CAA ATA CTC GGA TT-3') (CinnaGen Inc., Teheran, Iran).

PCR amplifications were carried out with a thermal cycler (Mastercycler gradient, Eppendorf, Hamburg, Germany) in 100 µl reaction volume. A 10 µl suspension of boiled bacterial cells was added to 90 µl of PCR mixture containing 2 mM MgCl, 20 pmol of each primer, 100 µM of each dNTP, 0.2 U of *Taq* DNA polymerase (CinnaGen Inc., Teheran, Iran), in 10 mM Tris-HCl (pH 9.0), 50 mM KCl and 0.1% Triton X-100. A denaturation step of 93°C for 2 min was followed by 37 cycles at 94°C for 1 min, 52°C for 2 min, and 72°C for 2 min, with a final extension step of 72°C for 10 min. Amplified DNA fragments were examined by electrophoresis in 2% agarose gel in TBE buffer (Maniatis *et al.*, 1982). Gels were stained with ethidium bromide and photographed under UV light (312 nm).

All of the 53 strains investigated (37 of *E. amylovora* and 16 of *P.s.* pv. *syringae*) were gram, oxidase, catalase, and pectinase negative (Table 1). In addition, *E. amylovora* isolates were able to utilize glucose under anaerobic conditions, produced ooze on pear slices, necrosis of leaves and shoots within 7-10 days, exudates and typical "shepherd's crook" in the shoots. Necrotic spots appeared on the leaves starting either from the edges or midribs and enlarged acquiring a triangular shape. *P. syringae* pv. *syringae* strains caused watery necrosis in the injured region of pear leaves after four days. No symptoms were shown by the controls.

Isolates of *E. amylovora* gave a positive response to the specific primers Ea1 and Ea2, which amplified a DNA fragment with the expected size of 937 bp, like that of the control strain CFBP 1430 (Fig. 1).

In conclusion, 37 strains of 53 were identified as *E. amylovora* and 16 as *P. syringae* pv. *syringae.* In pathogenicity tests no significant differences were observed in the development and severity of shoot blight among the various *E. amylovora* isolates. These results suggest that the bacterial strains isolated from different Iranian pear orchards or cultivars, similarly to what reported by Quamme and Bonn (1982), do not differ in virulence. This contrasts with the observations by others (Shaffer and Goodman, 1962; Goodman, 1973; Norelli, 1983) who detected a differential pathogenic behaviour. To the best of our knowledge this is the first report of fire blight of pear in northern Iran.

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